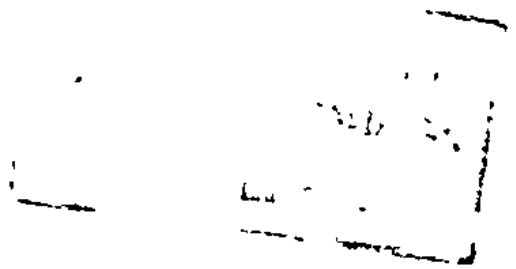
BIOCHEMICAL PREPARATIONS

Volume 1



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FOREWORD

No greater problem faces the scientist of today than keeping abreast of the voluminous literature. Investigations are handicapped by the necessity for the research man to spend an inordinate amount of time in the library. Accordingly, summaries and reviews of special fields of chemistry such as those that appear in *Chemical Reviews*, Ahren's *Sammlung*, or such books as *Preparative Organic Chemistry* or *Organic Reactions* are invaluable. Equally useful are summaries of specialized techniques.

Precise directions for the preparation of substances of important types also conserve the investigator's time. Often procedures in the literature are so highly condensed that essential details are not even mentioned. To obviate this difficulty *Organic Syntheses* was initiated in 1921 with the objective of affording the organic chemist procedures for the preparation of compounds frequently used in the laboratory. The reliability of the directions was established by checking each synthesis before publication in at least one laboratory other than that of the submitter. In 1939, the first volume of *Inorganic Syntheses* was published to offer checked directions for the synthesis of typical inorganic compounds.

A similar source of reliable procedures for the preparation of compounds of biochemical interest will be highly valuable. The initiation of *Biochemical Preparations* is timely and will be welcomed by biochemists and organic chemists. Greater effort may be required to produce these volumes because of the more numerous variables associated with the experiments involved. Variations in the composition of source materials, the frequently complicated purification procedures, and the difficulty encountered in evaluating purity of the products are factors that increase the complexity of assembling reliable procedures in this field. The success of these volumes is assured by the eminence of the

Editors. Chemists in general and especially the Advisory and Editorial Boards of *Organic Syntheses* look with enthusiasm upon this new enterprise.

ROGER ADAMS

PREFACE

For several years there has been a growing demand among biochemists for a "Preparations" series. The need for such a publication is well illustrated by the list (see page ix) of compounds of biochemical interest, the preparations of which have appeared in *Organic Syntheses*. *Biochemical Preparations* has been established to meet this need. An Advisory Board and an Editorial Board were formed and have held several conferences on questions of policy during the past two or three years. The process of establishing editorial policy will be a continuing one. The two major goals will be to provide authoritative, thoroughly checked preparations for substances used in biochemical research and to provide preparations illustrating manipulative techniques and methods that may be useful both to research workers and to students. The following principles regarding material to be published have been tentatively established.

There must be a readily available source of starting material for each preparation.

Preparations of substances which are available commercially at a low unit cost will be included only if they illustrate useful techniques or if they afford to students training in handling a natural product of a particular type.

Each preparation will be carefully checked in a laboratory other than that of the submitter. In this way it is hoped to avoid publication of procedures that cannot be readily duplicated.

In general, isolation methods will be emphasized, but if a compound used by biochemists is best obtained by synthesis the synthetic method will be published.

The format of *Biochemical Preparations* is similar to that of *Organic Syntheses*. However, the particular problems involved

in describing *isolation* procedures have necessitated certain modifications. The section "Properties and Purity of Product" has been added in the course of working up the material for Volume 1. This section should be helpful in indicating necessary precautions in experimental procedure and in evaluating the purity of the final product. The inclusion of footnotes at the bottom of the page and other differences also will be noted.

The Editors will be happy to receive material for Volumes 2 and 3, which are now being assembled. The manuscripts should be written in the style of Volume 1. The Editors will welcome comments or suggestions for increasing the usefulness of *Biochemical Preparations*.

It is a pleasure to acknowledge the advice and suggestions of the many biochemists who were consulted. We are particularly indebted to Dr. Roger Adams and other members of the *Organic Syntheses* Board for their helpful counsel.

H. E. C.

January, 1949

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3. Aconitic Acid, *Coll. Vol.* 2, 12
4 β-Alanine, *Coll. Vol* 2, 19; 27, 1
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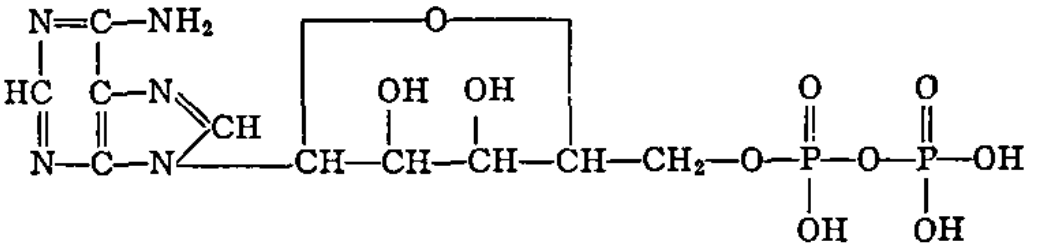
(ADP)



Isolated as the barium salt· $Ba_3(C_{10}H_{12}O_{10}N_5P_2)_2 \cdot 8H_2O$.
Mol wt. free acid 427 ($C_{10}H_{15}O_{10}N_5P_2$).

Submitted by G A LEPAGE, University of Wisconsin, Madison.
Checked by J. N. WILLIAMS, JR., and C. S. VESTLING, University of Illinois, Urbana.

I. Principle

Adenosine triphosphate is hydrolyzed to adenosinė diphosphate and inorganic phosphate by the enzyme adenosinetriphosphatase obtained from lobster or rabbit muscle. The adenosine diphosphate is isolated as the barium salt, then precipitated with *mercuric nitrate and reconverted to the barium salt.*

II. Starting Material

A. Sodium salt of adenosine triphosphate. A solution of 1.12 gm. of barium adenosine triphosphate (p. 7) in 30 ml. of cold 0.1 *N* hydrochloric acid is treated with 0.3 gm. of sodium sulfate. The precipitate is centrifuged and washed once with 10–15 ml. of 0.1 *N* hydrochloric acid. The supernatant and washings are combined and neutralized to *p*H 7 with 1 *N* potassium hydroxide. The solution is diluted to 100 ml., giving a 1% solution of adenosine triphosphate.

B. Adenosinetriphosphatase from lobster.[1] A live lobster is cut in half transversely, and the tail muscles are removed and cut into strips with scissors. The strips are suspended in 300 ml. of chilled 0.45% potassium chloride solution with slow mechanical stirring. The solution is changed every 15 minutes until 5 successive washings are made. The strips are then ready for use.

C. Adenosinetriphosphatase from rabbit muscle.[2] A rabbit is killed by a blow on the head. The leg muscles are removed at once, chilled, minced finely with scissors, and suspended in 5 volumes of 0.5 *M* potassium chloride-0.03 *M* sodium bicarbonate solution at 0°. The mixture is stirred slowly for 1 hour with frequent additions of solid sodium bicarbonate to maintain the *p*H at 7.0–7.5. The entire process is carried out in a cold room. The solid matter is centrifuged and the extract filtered through a Büchner funnel packed with paper pulp which has been saturated with the potassium chloride-sodium bicarbonate solution. The filtrate is adjusted to *p*H 6.8–7.0 with hydrochloric acid and diluted with 20 volumes of water. The precipitate is centrifuged and extracted twice with the potassium chloride-sodium carbonate solution to give a final volume equal to approximately half that of the original extract. Any insoluble matter is filtered through paper pulp saturated with the extracting fluid. The enzyme solution is dialyzed against the potassium chloride-sodium bicarbonate solution (*p*H 7) for 12–24 hours with several changes of solution.

This enzyme preparation is stable for several days in the cold. The activity varies considerably from one preparation to another. An amount should be used which will effect the conversion of adenosine triphosphate to the diphosphate in 20–40 minutes at 37°.

III. Procedure

To 100 ml. of the 1% adenosine triphosphate solution are added 25–35 gm. of muscle strips from lobster tail. The mixture is stirred slowly for 20 minutes at 20°. The strips are filtered, and

[1] K. Lohmann, *Biochem. Z.*, 282, 109 (1935).
[2] K. Bailey, *Biochem. J.*, 36, 121 (1942).

the easily hydrolyzable phosphorus [3] is determined. If this has not decreased 50% a further incubation with the muscle strips is necessary. The muscle strips may be used to treat several batches of adenosine triphosphate. The final solution is freed of muscle by filtration and adjusted to *p*H 6.8 with hydrochloric acid. Five milliliters of 1 *M* barium acetate (excess) are added, and the mixture is chilled. The precipitate is centrifuged and redissolved in a minimum of cold 0.2 *N* nitric acid. The cold solution is treated with Lohmann's reagent (see adenosine triphosphate preparation, p. 7) and the precipitate is centrifuged, suspended in 75 ml. of water, and decomposed with hydrogen sulfide. The mercuric sulfide is centrifuged and washed with 15 ml. of 0.2 *N* nitric acid. The solution and washings are combined and aerated free of hydrogen sulfide. Final traces of mercuric sulfide remaining in suspension can be removed by addition of barium acetate and a few milliliters of 0.1 *N* sulfuric acid. Centrifugation of the barium sulfate carries down the mercuric sulfide. The solution is adjusted to *p*H 6.8, and 1 volume of 95% ethanol and an excess of barium acetate are added. The solution is chilled, and barium adenosine diphosphate precipitates. The recovery is poor if alcohol is omitted since the barium salt is much more soluble in pure solution than when inorganic phosphate is present to precipitate with it. The precipitate is washed successively with 5 times its volume of 50% ethanol (neutralized), 75% ethanol, 95% ethanol, and ether. It is dried in vacuum over calcium chloride or sulfuric acid. The yield of barium salt of adenosine diphosphate tetrahydrate is about 0.3 gm. (40%).

If rabbit muscle adenosinetriphosphatase is employed an amount sufficient to complete the hydrolysis in 20–40 minutes should be used. The incubation is carried out at 37° (instead of 20° as for lobster muscle). After the digestion is complete the solution is deproteinized by addition of trichloroacetic acid to a final concentration of 10%. The precipitate is centrifuged, and

[3] W. W. Umbreit, R. H. Burris, and J. F. Stauffer, *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, p. 166, Burgess Publishing Co., 1945.

the solution is worked up as described for the lobster-muscle preparation. Lobster muscle, if available, is preferable as a source of enzyme.

IV. Properties and Purity of Product

The purity of the preparations can be checked by analyses for nitrogen, ribose (orcinol reaction), total phosphorus, easily hydrolyzable phosphorus,[3] and inorganic phosphorus.[4] Adenosine diphosphate should give a ratio of 2:1 for total organic phosphorus to easily hydrolyzable phosphorus. Preparations obtained by this method are 95–98% pure and contain about 0.1% of inorganic phosphorus.

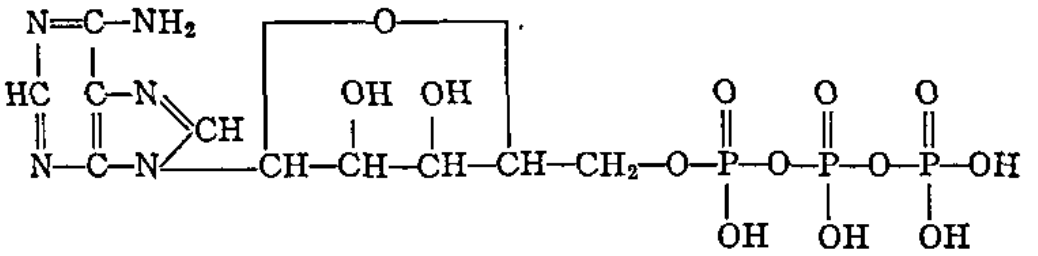
V. Methods of Preparation

Adenosine diphosphate can be prepared by the enzymatic hydrolysis of adenosine triphosphate with adenosinetriphosphatase preparations from lobster [1] or rabbit muscle.[2]

[4] W. W. Umbreit, R. H. Burris, and J. F. Stauffer, *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, p. 165, Burgess Publishing Co., 1945.

ADENOSINE TRIPHOSPHATE

(ATP)



Isolated as the barium salt: $Ba_2(C_{10}H_{12}O_{13}N_5P_3)\cdot 4H_2O$.
Mol. wt. free acid 508.2 ($C_{10}H_{16}O_{13}N_5P_3$).

Submitted by G. A. LEPAGE, University of Wisconsin, Madison.
Checked by J. N. WILLIAMS, JR, and C. S. VESTLING, University of Illinois, Urbana.

I. Principle

Adenosine triphosphate is present in resting mammalian skeletal muscle to the extent of 350–400 mg. per cent. It is hydrolyzed to adenosine diphosphate by the enzyme adenosinetriphosphatase when muscle is stimulated. However, a good yield of adenosine triphosphate is obtained by inhibiting the enzyme with magnesium [1] and extracting the muscle tissue with acid. Adenosine triphosphate, other phosphate esters, and inorganic phosphate are precipitated as the barium salts. The precipitate is dissolved in dilute nitric acid, and adenosine triphosphate is precipitated with mercuric nitrate. The precipitate is decomposed with hydrogen sulfide, and the desired product is isolated as the barium salt.

II. Starting Material

Mammalian muscle is the best known source of adenosine triphosphate. Beef muscle has been used because of availability in quantity [2] but gives poor yields on isolation because it cannot

[1] K. P. DuBois, H G. Albaum, and V R. Potter, *J Biol. Chem*, **147**, 699 (1943).
[2] V. L. Koenig and J. J. Svarz, *Arch. Biochem.*, **6**, 269 (1945).

be handled rapidly enough to limit enzymatic decomposition. The rabbit appears to be the ideal animal for the source of muscle. Lean, male rabbits are preferable. Five hundred to seven hundred grams of muscle are obtained from the back and hind leg musculature of a 3-kg. rabbit.

It is possible to prepare large amounts of the compound with the minimum of work by pooling the extracts from muscles of several rabbits. However, the process should be carried as far as extraction of the muscles and neutralization of the extracts without delay.

III. Procedure

A rabbit is brought to a completely paralyzed state and finally anesthetized by a series of small intraperitoneal injections of magnesium sulfate solution (25% $MgSO_4$ or 51% $MgSO_4 \cdot 7H_2O$). The first injection consists of 250 mg. per kg. of body weight followed by a 125 mg. per kg. dose every 10 minutes until complete anesthesia is reached. The animal should be flaccid with no inhibition of respiration or cyanosis and with the muscles remaining completely relaxed throughout the injection period. This condition is usually reached in 30 minutes, when the animal is killed by decapitation.

The muscles from the back and the hind legs are cooled in cracked ice until all have been removed and trimmed free of fat. The trimmed muscles are quickly weighed and ground (preferably in a cold room), in an equal volume of ice-cold 10% trichloroacetic acid, by means of a Waring blendor. The precipitated muscle proteins and fiber masses are separated from the extract by pressing through cheesecloth. The residue is re-extracted in the Waring blendor with an equal volume of 5% trichloroacetic acid and again pressed through cheesecloth. The combined extracts are filtered on a Büchner funnel and brought to *p*H 6.8 (bromthymol blue indicator) with 10 *N* sodium hydroxide (about 0.4 ml. per liter of filtrate is required). The dibarium salt of the adenosine triphosphate, together with any adenosine diphosphate, hexosediphosphate, phosphoglyceric acid, and inorganic phosphate, is precipitated by the addition of 3.0 ml. of 2 *M* barium

acetate per 100 gm. of muscle (excess). The suspension is chilled for 30 minutes and centrifuged in the cold. The supernatant is discarded, and the precipitate is dissolved in 0.2 *N* nitric acid (30–50 ml. per 100 gm. muscle). A small insoluble residue is filtered or centrifuged and discarded.

The solution is treated with mercuric nitrate (Lohmann's reagent: 100 gm. of $Hg(NO_3)_2 \cdot 8H_2O$, 25 ml. of H_2O, and 25 ml. of concentrated HNO_3), using 0.6–1.0 ml. per 100 gm. of muscle. The mixture is cooled in the refrigerator for 15 minutes, the mercury salts are centrifuged, and the supernatant is discarded. The precipitate is suspended in a small volume of water and decomposed with hydrogen sulfide. The mercuric sulfide is centrifuged, washed once with approximately 5 times its volume of 0.2 *N* nitric acid, and discarded. The combined supernatant and washings are aerated free of hydrogen sulfide. The solution is neutralized to *p*H 6.8, and 2.0 ml. of 2 *M* barium acetate per 100 gm. of muscle are added. The suspension is chilled and centrifuged in the cold. The precipitate is washed successively (using 6–8 times the volume of the wet precipitate) with: (1) 1% barium acetate at *p*H 6.8; (2) 50% ethanol; (3) 75% ethanol; (4) 95% ethanol; (5) ether. The precipitate is dried in a vacuum desiccator over calcium chloride or sulfuric acid.

A purer product is obtained by the following alternative procedure: After precipitation of the barium salt of adenosine triphosphate, but before washing with alcohol and ether, the precipitate is dissolved in 0.2 *N* nitric acid and the process of precipitation as mercury salt, decomposition with hydrogen sulfide, and reconversion to barium salt is repeated. After centrifuging and filtering the mercuric sulfide precipitate, a trace of mercuric sulfide often remains suspended in the solution. This contaminates the final product and may be damaging to enzyme systems. It can be removed, before neutralizing the acid solution, by addition of a small amount of barium acetate and 3–5 ml. of 0.1 *N* sulfuric acid. The small barium sulfate precipitate is centrifuged and carries down with it the last traces of mercuric sulfide.

Yields obtained from rabbits anesthetized with magnesium

sulfate generally exceed 3.5 gm. of barium adenosine triphosphate per kilogram of muscle.

For use the barium salt is converted to the sodium or potassium salt. Eighty-five milligrams of barium salt are dissolved in 5 ml. of 0.1 *N* hydrochloric acid, and 35 mg. of sodium sulfate are added. The barium sulfate is centrifuged and washed once with 2 ml. of 0.1 *N* hydrochloric acid. (Adsorption of adenosine triphosphate on barium sulfate takes place above *p*H 3 but is minimal in 0.1 *N* hydrochloric acid.) The supernatant and wash are mixed and neutralized. If chlorides are undesirable the barium salt is decomposed with a slight excess of 0.1 *N* sulfuric acid. The barium sulfate is removed and washed thoroughly. The supernatant and washings are then neutralized with sodium hydroxide.

IV. Properties and Purity of Product

The product can be tested for purity by analysis for nitrogen, ribose (orcinol reaction), or phosphorus, and by the ratio of easily hydrolyzable phosphorus to total organic phosphorus. The only impurities likely to be present in these preparations are barium salts of orthophosphoric acid and adenosine diphosphate. Measurement of inorganic phosphorus[1] and of the ratio of phosphorus hydrolyzable in 1.0 *N* hydrochloric acid at 100° in 7 minutes[1] to total organic phosphorus[1] therefore serve as the criteria of purity. This ratio is 3:2 for pure adenosine triphosphate since only two of the phosphate groups are hydrolyzed with the 1.0 *N* hydrochloric acid. Preparations obtained by this method consistently contain 0.14% inorganic phosphorus (probably as $Ba_3(PO_4)_2$) and are 98% pure.

Adenosine triphosphate is stable at 0° for several hours in acid solutions (e.g., 7% trichloroacetic acid). At *p*H 6.8–7.4 it is stable in solution (0°) for approximately 1 week. There is a slow release of inorganic phosphorus and some danger of microbial action.

[1] W. W. Umbreit, R. H. Burris, and J. F. Stauffer, *Manometric Techniques and Related Methods for the Study of Tissue Metabolism*, pp. 165–166, Burgess Publishing Co., 1945.

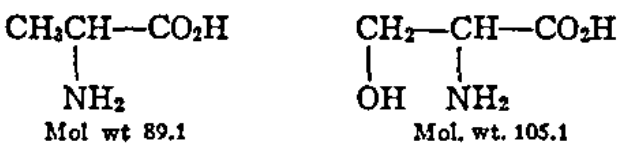
V. Methods of Preparation

Adenosine triphosphate is most conveniently prepared from fresh muscle tissue.[4] Frog muscle [4b] and rabbit muscle [4b,c,e] give satisfactory results. Beef muscle gives considerably poorer yields.[2] Fresh horse muscle has also been employed.[5]

Adenosine triphosphate may be isolated and purified tnrough the barium,[4b,d,e] calcium,[4a,c] or silver salts [4c] or by precipitation with mercuric acetate in dilute acetic acid [4a,c,d] or mercuric nitrate in nitric acid.[4b,e] The present method is essentially that of Needham.[4e]

The enzymatic conversion of adenosine triphosphate to adenosine diphosphate is described in the preceding preparation (p. 1). Kerr [6] has described the preparation of adenylic acid by hydrolysis of adenosine triphosphate with barium hydroxide.

L-ALANINE and L-SERINE



Mol wt 89.1 Mol. wt. 105.1

Submitted by WILLIAM H. STEIN and STANFORD MOORE, the Rockefeller Institute for Medical Research, New York.
Checked by F. R. VAN ABEELE and H. E. CARTER, University of Illinois, Urbana.

I. Principle

Acid hydrolysis of silk fibroin yields a solution containing approximately 44 gm. of glycine, 30 gm. of alanine, 15 gm. of serine, and 12 gm. of tyrosine per 100 gm. of protein.[1] Other amino acids

[4] (*a*) C. H. Fiske and Y. Subbarow, *Science*, 70, 381 (1929) (*b*) K. Lohmann, *Biochem. Z*, 233, 460 (1931); 254, 381 (1932). (*c*) H. K. Barrenscheen and W. Filz, *Biochem Z.*, 250, 281 (1932). (*d*) S. E. Kerr, *J. Biol. Chem*, 139, 121 (1941). (*e*) D. M. Needham, *Biochem J.*, 36, 113 (1942).

[5] K. Bailey, *Biochem J*, 36, 121 (1942)

[6] S E. Kerr, *J. Biol. Chem*, 139, 131 (1941)

[1] W. H. Stein, S Moore, and M. Bergmann, *J. Biol. Chem*, 154, 191 (1944).

are present in relatively small quantities. Tyrosine is removed from the hydrolysate by direct crystallization, glycine as a salt of 5-nitronaphthalene-1-sulfonic acid, alanine as a salt of azobenzene-*p*-sulfonic acid, and serine as a salt of *p*-hydroxyazobenzene-*p'*-sulfonic acid.[2] In order to obtain pure preparations *the amino acids must be isolated in the order named.* The hydrolysate must be almost free of glycine prior to the use of azobenzene-*p*-sulfonic acid, and both glycine and alanine must be removed before the addition of *p*-hydroxyazobenzene-*p'*-sulfonic acid. The free amino acids are readily obtained from the salts, and provision is made for the recovery of the major portion of the sulfonic acid reagents in a form suitable for reuse.

II. Starting Material

Silk fibroin. Technically degummed Japanese white silk can be obtained from the U. S. Testing Laboratory, Hoboken, New Jersey.

III. Procedure

A. Hydrolysis of fibroin and isolation of sulfonates. Degummed Japanese white silk (525 gm., containing 5% moisture) is suspended in 1.5 l. of concentrated hydrochloric acid in a 12-l. flask, and the mixture is refluxed for 8 hours on a sand bath. The hydrolysate is concentrated to a syrup under reduced pressure (water pump); 250 ml. of water are added, and the solution is again concentrated to a syrup. The operation is repeated twice more. The final residue is diluted to a volume of about 2 l., and to it is added slowly, with vigorous stirring, a hot solution of 1.3 kg. of lead acetate trihydrate[3] in 2 l. of water. The mixture is cooled to room temperature, and the precipitate of lead chloride is filtered on a large Büchner funnel. The precipi-

[2] W. H. Stein, S. Moore, G. Stamm, C. Chou, and M. Bergmann, *J. Biol. Chem.*, 143, 121 (1942).

[3] The lead and barium salts used throughout this preparation are analytical reagent grade.

tate is washed on the filter with three 300-ml. portions of water and then discarded.[4] The filtrate and washings, in a 12-l. flask, are stirred vigorously while a stream of hydrogen sulfide is passed in.[5] The lead sulfide is filtered on a pad of celite [6] on a large Büchner funnel and washed on the filter with 200 ml. of water. The yellow filtrate and washings [7] are concentrated under reduced pressure to a heavy syrup in order to remove the bulk of the acetic acid. Tyrosine precipitates during the concentration. The syrup is diluted to 1 l. with water, and the tyrosine is filtered and washed on the filter with 150 ml. of water. The filtrate and washings are diluted to 2 l.[8]

In order to remove glycine, 860 gm. of 5-nitronaphthalene-1-sulfonic acid (see p. 20) are added to the hydrolysate, and the mixture is heated, with stirring, to effect a clear solution. The mixture is cooled and placed in the refrigerator overnight. The crystalline glycine salt is filtered on a Büchner funnel, washed on the filter with three 400-ml. portions of ice water, and recrystallized from 2.7 l. of water. The yield of air-dried product is 780–800 gm. The salt is set aside for recovery of the sulfonic acid.

To the combined filtrate and washings from which glycine has been removed are added 400 ml. of methyl cellosolve and 640 gm. of azobenzene-*p*-sulfonic acid (see p. 15). The mixture is stirred and heated to dissolve the sulfonic acid. The solution is cooled and placed in a refrigerator overnight. The precipitated alanine salt is filtered and washed on the funnel with two 400-ml. portions of ice water. The alanine azobenzene sulfonate is recrys-

[4] The filtrate, after removal of the lead chloride, should give no more than a faint precipitate upon the addition of a little lead acetate solution.

[5] The lead sulfide precipitate coagulates when sufficient hydrogen sulfide has been passed into the solution. The use of insufficient hydrogen sulfide leads to a colloidal suspension.

[6] Hyflo Super-Cel or a similar material may be used. The filter aid may be stirred into the mixture before filtering if the filtration is too slow.

[7] The filtrate after removal of lead sulfide should be tested for lead ions by bubbling hydrogen sulfide into a small sample.

[8] Unless used immediately, all amino acid solutions should be stored in the refrigerator to minimize growth of bacteria and molds.

tallized from 2600 ml. of hot water [9] and dried in air. The yield is 445–460 gm. The product is set aside for recovery of both the azobenzenesulfonic acid and alanine.

To the filtrate and washings from which alanine has been removed is added a hot solution of 290 gm. of barium acetate dihydrate in water. The precipitate [10] is filtered on a mat of celite and washed on the filter with three 500-ml. portions of water. To the filtrate and washings 30 ml. of concentrated sulfuric acid are added, and the barium sulfate is filtered on a pad of celite. The precipitate is washed with two 200-ml. portions of water and discarded. The filtrate and washings (which should contain no barium and only a slight excess of sulfuric acid) are concentrated under reduced pressure (water pump) to a thin syrup. The syrup is diluted to 2.2 l. with water, and 275 ml. of methyl cellosolve and 320 gm. of *p*-hydroxyazobenzene-*p'*-sulfonic acid (see p. 17) are added. The mixture is heated and stirred until a clear solution results. The solution is cooled and placed in the refrigerator overnight. The serine hydroxyazobenzene sulfonate is filtered on a Büchner funnel and washed on the filter with cold water until most of the deep red color has disappeared and the precipitate is orange or yellow. The serine salt is recrystallized twice, once from 1050 ml., and the second time from 850 ml. of water. The yield of recrystallized salt is 190–215 gm.

B. Isolation of the amino acids and recovery of the sulfonic acids. 1. *Alanine and azobenzenesulfonic acid.* Four hundred and fifty grams of alanine azobenzene sulfonate are dissolved in 3.7 l. of hot water, and a hot solution of 340 gm. of barium acetate dihydrate in about 750 ml. of water is added. From the cooled mixture the precipitate is removed on a Büchner funnel and

[9] Frequently small quantities of inorganic salts of the sulfonic acid are present and will appear as finely divided material which will not pass into solution. This insoluble material is removed by filtering the hot solution through a folded filter. After the filter has been washed with 500 ml. of hot water, the filtrate and washings are stored in the refrigerator overnight for crystallization.

[10] This precipitate consists of a mixture of the barium salts of nitronaphthalene and azobenzenesulfonic acids. These substances must be removed in order to reduce the acidity of the hydrolysate prior to the addition of *p*-hydroxyazobenzene-*p'*-sulfonic acid, which is sparingly soluble in strongly acid solution.

washed on the filter with three 750-ml. portions of water. The precipitate is set aside for recovery of the sulfonic acid.[11]

To the filtrate and washings 40 ml. of concentrated sulfuric acid are added, and the solution is exactly freed of barium and sulfate ions. Acid-washed charcoal is added, and the barium and charcoal are filtered on a mat of celite. The filtrate, which should be colorless or faintly yellow, is concentrated to dryness under reduced pressure (water pump). The residue is dissolved in 400 ml. of water, and the solution, if it is not colorless, is warmed and decolorized with acid-washed charcoal. To the warm solution 1.4 l. of hot absolute ethanol are added with stirring. The mixture is placed in the refrigerator overnight. The L-alanine is filtered, washed on the filter with 100 ml. of cold 80% ethanol, and recrystallized from 300 ml. of hot water by adding 1.2 l. of ethanol. The yield of L-alanine is 85–90 gm.[12]

For recovery of azobenzenesulfonic acid, the barium salt is suspended in 400 ml. of alcohol to which 45 ml. of concentrated sulfuric acid have been added. The mixture is stirred mechanically and heated to boiling on a hot plate. The barium sulfate is filtered on a mat of celite, washed with 100–200 ml. of water, and discarded. To the warm filtrate and washings 400 ml. of concentrated hydrochloric acid are added, and the mixture is placed in the refrigerator overnight. The azobenzene-*p*-sulfonic acid is filtered,[13] washed with 100–150 ml. of cold 6 *N* hydrochloric acid, and dried in air. The yield is 360–380 gm. (about 60% of the sulfonic acid originally used).

2. *Serine and p-hydroxyazobenzene-p′-sulfonic acid.* One hundred and eighty-five grams of serine-*p*-hydroxyazobenzene-*p′*-sulfonate are dissolved in 700 ml. of hot water, and a hot solution of 135 gm. of barium acetate dihydrate in 300 ml. of water is added.

[11] The barium salts of the sulfonic acids should not be allowed to dry out since the dry material is more difficult to work up for the recovery of the sulfonic acids.

[12] Additional quantities (from 10 to 25 gm.) of alanine may be recovered from the mother liquors by concentration in vacuum. This procedure is particularly worth while if the preparation is carried out on a larger scale The alanine thus obtained may require one or two more crystallizations to attain purity.

[13] The filtration of azobenzene-*p*-sulfonic acid is very slow and should be done in a cold room.

The mixture is cooled to room temperature, and the barium salt is filtered, washed with 400 ml. of water, and saved for the recovery of the sulfonic acid.[11]

The pale yellow filtrate and washings are freed exactly of barium and sulfate ions. Acid-washed charcoal is added, and the barium sulfate and charcoal are filtered on a mat of celite and washed with 100 ml. of water. The filtrate and washings are concentrated to dryness under reduced pressure (water pump). The residue is dissolved in 150 ml. of warm water, and the solution is decolorized again with charcoal. To the solution 1.2 l. of warm absolute ethanol are added, and the mixture is stored in the refrigerator overnight. The serine is filtered, washed with 100 ml. of cold 80% ethanol, and recrystallized from a solution in 150 ml. of water by the addition of 1.2 l. of ethanol, as before. The yield of L-serine is 36–40 gm.

For recovery of the *p*-hydroxyazobenzene-*p'*-sulfonic acid, the barium salt obtained above is converted to the free sulfonic acid in the manner described in the preparation of the sulfonic acid (see p. 17). The yield of sulfonic acid suitable for reuse is 125–135 gm. (about 40% of the sulfonic acid originally employed).

3. *5-Nitronaphthalene-1-sulfonic acid.* For recovery of 5-nitronaphthalene-1-sulfonic acid, the glycine salt is converted to the free acid over the barium salt in the manner already described (see p. 20). The yield of sulfonic acid suitable for reuse is 650–680 gm. (80% of the quantity originally used).

IV. Properties and Purity of Product

The L-alanine and L-serine obtained by this procedure give correct elementary analyses. Typical optical rotations are:

L-Alanine: $[\alpha]_D^{20} = +8.5$ (9.3% solution of alanine hydrochloride in water).[2,14,15]

L-Serine: $[\alpha]_D^{26} = +14.8$ (10% solution in 2 *N* hydrochloric acid).[2]

[11] (*a*) E. Fischer, *Ber.*, **32**, 2451 (1899); (*b*) J. B. Dalton and C. L. A. Schmidt, *J. Biol. Chem.*, **103**, 549 (1933); (*c*) H. M. Huffman and H. Borsook, *J. Am. Chem. Soc.*, **54**, 4297 (1932).

[15] E. Fischer, *Ber.*, **39**, 453 (1906).

V. Methods of Preparation

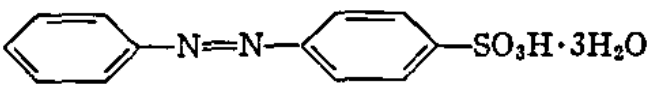
There are no other satisfactory procedures for the isolation of L-alanine and L-serine from natural products, although L-alanine has been obtained from silk by the Fischer ester method [15] and DL-serine has been prepared from sericin.[16]

DL-Alanine has been resolved through the brucine salts of the benzoyl derivatives,[14a] through the menthoxyacetyl derivatives,[17] and more recently through a combination of strychnine and brucine salts of the benzoyl derivatives.[18]

DL-Serine has been resolved through the quinine salts of the *p*-nitrobenzoyl derivatives.[19]

DL-Alanine and DL-serine are both readily available by synthetic methods.[20]

AZOBENZENE-*p*-SULFONIC ACID TRIHYDRATE



Mol. wt. 316.3

Submitted by WILLIAM H. STEIN and STANFORD MOORE, the Rockefeller Institute for Medical Research, New York.

Checked by F. R. VAN ABEELE and H. E. CARTER, University of Illinois, Urbana.

I. Principle

Azobenzene is sulfonated with fuming sulfuric acid, and the crude product is recrystallized from ethyl acetate and from aqueous-ethanolic hydrochloric acid.

II. Procedure

Azobenzene (300 gm.) is pulverized by grinding in a mortar in a hood. In a 5-l. round-bottomed flask, equipped with an

[16] F. S. Daft and R. D. Coghill, *J. Biol. Chem.*, 90, 341 (1931).

[17] D. F. Holmes and R. Adams, *J. Am. Chem. Soc.*, 56, 2093 (1934).

[18] E. Pacsu and J. W. Mullen, *J. Biol. Chem.*, 136, 335 (1940).

[19] E. Fischer and W. A. Jacobs, *Ber.*, 39, 2942 (1906).

[20] Cf. Dunn and Schmidt, *Chemistry of the Amino Acids and Proteins*, Chapter 2.

efficient stirrer and surrounded by a water bath at room temperature, are placed 900 ml. of fuming sulfuric acid (15% sulfur trioxide; Nordhausen).[1] The powdered azobenzene is added slowly with stirring, and each portion is allowed to dissolve before the next is added. The temperature of the reaction mixture is maintained below 50°. The addition of the azobenzene requires about 30 minutes. The mixture is then heated on a steam bath at 75–80° for 5 minutes. The mixture is cooled to 50° and poured slowly and with vigorous stirring onto 5 l. of ice.[2] To the hot solution 1 l. of concentrated hydrochloric acid is added, and the mixture is cooled and placed in the refrigerator overnight. The orange crystals of azobenzene-*p*-sulfonic acid are filtered on a 25-cm. Büchner funnel.[3] The filtration is very slow. The precipitate is sucked as dry as possible and while still moist is dissolved in 1 l. of hot water. The solution is filtered, and 1 l. of 95% ethanol and 1 l. of concentrated hydrochloric acid are added to the filtrate. The mixture is cooled overnight in the refrigerator. The precipitate is filtered on a 25-cm. Büchner funnel, washed on the filter with a cold mixture of 300 ml. each of water, alcohol, and concentrated hydrochloric acid, and air-dried. The dry acid is dissolved in 1 l. of hot ethyl acetate, and the solution is filtered and cooled in the refrigerator overnight. The precipitate is filtered, air-dried, and recrystallized from a mixture of 600 ml. of 50% ethanol and 400 ml. of concentrated hydrochloric acid. The mixture is cooled overnight, and the precipitate is filtered and air-dried. The yield of azobenzene-*p*-sulfonic acid trihydrate is 385–405 gm. (70–73%).

[1] A good hood should be employed when working with fuming sulfuric acid, and glasses and rubber gloves should be worn. If acid comes in contact with the skin, it should be wiped off and the area flooded with water immediately. Glassware which has contained fuming sulfuric acid should be rinsed by the rapid addition of large amounts of cold water. (Beware spattering.)

[2] Toward the end of the addition the mixture becomes hot, and care must be taken to minimize spattering.

[3] The filtration should be performed in a cold room (+5°). Only glass or porcelain equipment should be used in contact with the sulfonic acid; this acid has a powerful solvent action on metal. Glass wool or glass cloth may be used to advantage in filtering these highly acid solutions.

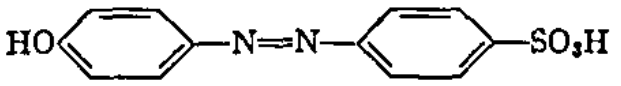
III. Properties and Purity of Product

The product gives correct analytical data and is satisfactory for use in the preparation of L-alanine and L-serine from silk fibroin (p. 9).

IV. Methods of Preparation

Azobenzene-*p*-sulfonic acid has been prepared by the sulfonation of azobenzene [4] and by the reduction of nitrobenzene with sulfite waste liquor.[5]

p-HYDROXYAZOBENZENE-*p'*-SULFONIC ACID



Mol. wt. 278.2

Submitted by WILLIAM STEIN and STANFORD MOORE, the Rockefeller Institute for Medical Research, New York.
Checked by P A. TAVORMINA and H. E. CARTER, University of Illinois, Urbana.

I. Principle

Sulfanilic acid is diazotized,[1] and the *p*-benzenediazonium sulfonate is coupled with phenol in an alkaline solution. The *p*H of the final solution is lowered to 7 with hydrochloric acid, causing the sodium salt of the sulfonic acid to precipitate. The product is converted to the sulfonic acid through the barium salt.

II. Procedure

A. Diazotization of sulfanilic acid.[1] To a solution of 53 gm. (0.50 mole) of anhydrous sodium carbonate in 1 l. of water is

[4] P. Griess, *Ann*, 154, 208 (1870), P. Ruggli and M. Stauble, *Helv. Chim. Acta*, 24, 1080 (1941); W. H Stein, S Moore, G. Stamm, C. Chou, and M. Bergmann, *J. Biol. Chem.*, 143, 121 (1942).

[5] I. A Pearl, *J. Org Chem*, 9, 424 (1944).

[1] L. F. Fieser, *Org Syntheses* Coll. Vol 2, 35 (1943)

added 173 gm. (1.0 mole) of sulfanilic acid (or 209 gm. of the dihydrate), and the mixture is cooled to 0–5°. A solution of 74 gm. (1.08 moles) of sodium nitrite in 200 ml. of water is added, and the resulting solution is poured immediately into a mixture of 1.2 kg. of cracked ice and 212 ml. (2.5 moles) of concentrated hydrochloric acid (sp. gr. 1.18). The *p*-benzenediazonium sulfonate separates within a few minutes, and the suspension is stirred at 0–5° for 30–45 minutes.

B. Coupling with phenol. A solution of 94.0 gm. (1.0 mole) of phenol in 500 ml. of water containing 98.0 gm (2.52 moles) of sodium hydroxide is cooled to 0–5°. The solution is stirred mechanically, and to it is added the suspension of the diazonium sulfonate in a slow steady stream. The temperature of the reaction mixture is maintained below 5° by the addition of ice from time to time (100–300 gm. required). A small amount of the sodium salt of the sulfonic acid may separate toward the end of the reaction. The *p*H of the reaction mixture is 9–10 after the diazonium sulfonate has been added. The mixture is acidified to *p*H 7.0–7.2 by the addition of 6 *N* hydrochloric acid (about 120 ml. are required), warmed to 80–85°, and allowed to cool. The precipitate is filtered with suction and air-dried. This material is satisfactory for use in the next step. If purified sodium salt is desired the crude moist precipitate is transferred to a beaker and enough water is added to effect complete solution at 85–90° (about 3 l.). The hot solution is allowed to cool slowly to room temperature, and the orange-yellow crystals are filtered and air-dried, giving 272–290 gm. (81–86% yield) of sodium-*p*-hydroxyazobenzene-*p'*-sulfonate dihydrate.

C. Conversion to *p*-hydroxyazobenzene-*p'*-sulfonic acid. Four hundred and eighty grams of the sodium salt are dissolved in hot water, and a solution of 380 gm. of barium acetate monohydrate in 300 ml. of hot water is added with vigorous stirring. The mixture is allowed to cool to room temperature, and the barium salt is filtered on a large Büchner funnel and washed on the filter with 3 l. of water. The wet precipitate [2] is suspended in a mixture

[2] The precipitate should not be allowed to dry since subsequent suspension and decomposition with sulfuric acid are rendered more difficult.

of 3.3 l. of water and 750 ml. of methyl cellosolve to which 75 ml. of concentrated sulfuric acid have been added. The mixture is stirred mechanically and heated to boiling to decompose the barium salt. The barium sulfate is filtered through a pad of celite, 1.5 l. of concentrated hydrochloric acid are added to the filtrate, and the mixture is cooled in a refrigerator overnight. The precipitate is filtered and washed with 1 l. of cold 6 *N* hydrochloric acid. The yield of air-dried *p*-hydroxyazobenzene-*p'*-sulfonic acid is 360–380 gm. (90–95%).

III. Properties and Purity of Product

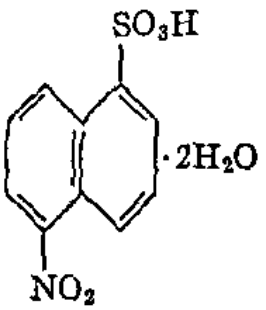
The product gives the correct analytical data for *p*-hydroxyazobenzene-*p'*-sulfonic acid and is suitable for use in the preparation of L-alanine and L-serine (p. 9).

IV. Methods of Preparation

p-Hydroxyazobenzene-*p'*-sulfonic acid has been prepared by the coupling of diazotized sulfanilic acid with phenol [3] and with *p*-hydroxybenzoic acid.[4] It has also been obtained from *p*-aminoazobenzene-*p'*-sulfonic acid through the diazonium salt [5] and by sulfonation of *p*-hydroxyazobenzene.[6] The present procedure is essentially that described by Fieser [1] for the diazotization of sulfanilic acid and coupling of the diazonium salt with β-naphthol.

[3] P Griess, *Ber.*, II, 2191 (1878); K H Engel, *J Am Chem. Soc.*, 51, 2986 (1929)
[4] H. Limpricht, *Ann*, 263, 224 (1891).
[5] P. Griess, *Ber.*, 15, 2183 (1882)
[6] Tschurvinsky, *J. Russ. Phys Chem. Soc*, 5, 215 (1873).

5-NITRONAPHTHALENE-1-SULFONIC ACID *DIHYDRATE*



Mol. wt. 289.2

Submitted by WILLIAM H. STEIN and STANFORD MOORE, the Rockefeller Institute for Medical Research, New York.
Checked by F. R. VAN ABEELE and H. E. CARTER, University of Illinois, Urbana.

I. Principle

α-Nitronaphthalene is treated with hot concentrated sulfuric acid, and the sulfonic acid is isolated and purified as the insoluble glycine salt. This is converted to the barium salt, from which the free sulfonic acid is obtained by treatment with sulfuric acid.

II. Procedure

Five hundred grams of α-nitronaphthalene [1] and 1 l. of concentrated sulfuric acid are heated on a steam bath for 6 hours. The mixture is cooled to room temperature and is poured with stirring into 10 l. of ice water. To the dark suspension is added 40 gm. of charcoal (Darco G-60), and the mixture is filtered through a pad of charcoal and Hyflo Super-Cel (Johns-Manville). Three hundred and seventy-five grams of glycine are dissolved in the filtrate, and the solution is cooled in the refrigerator overnight. The glycine salt of 5-nitronaphthalene-1-sulfonic acid is filtered and air-dried. The yield is 180–220 gm. (19–23%). For purification 200 gm. of the salt are suspended in 4 l. of boiling

[1] Eastman technical grade.

ethanol, and water is added cautiously until the salt dissolves (only a small quantity is required). The solution is clarified with acid-washed charcoal (Darco KG or G-60) and is filtered through a pad of Hyflo Super-Cel on a steam funnel. The pale yellow glycine salt crystallizes in square plates when the filtrate is cooled in a refrigerator for several hours. The salt is filtered [2] and washed with ethanol and ether. It is recrystallized from water employing 35 ml. per 10 gm. of salt. (The filtrates may be used in recrystallizing successive batches of salt.) The yield of purified glycine salt of 5-nitronaphthalene-1-sulfonic acid is 150–165 gm. (16–18%).

The glycine salt (150 gm.) is dissolved in 500 ml. of hot water. A hot solution of 125 gm. of barium acetate dihydrate (analytical reagent) (or an equivalent amount of the monohydrate) in 275 ml. of water [3] is added, and the mixture is allowed to cool to room temperature. The precipitate is filtered using a large Büchner funnel and is washed thoroughly on the filter with 800–1000 ml. of water. The filtrate is discarded. The moist precipitate [4] is suspended in 275 ml. of water to which 15 ml. of concentrated sulfuric acid have been added. The mixture is stirred and heated to boiling on the hot plate. The barium sulfate is removed by filtering the solution through a pad of Hyflo Super-Cel, and the precipitate is washed on the filter with about 50 ml. of water. To the warm filtrate 275 ml. of concentrated hydrochloric acid are added, and the mixture is cooled in the refrigerator overnight. The sulfonic acid, which crystallizes in pale yellow needles, is filtered, washed with 50 ml. of ice-cold 6 *N* hydrochloric acid, and air-dried. The yield of 5-nitronaphthalene-1-sulfonic acid dihydrate is 120–130 gm. (14–16% yield calculated on the α-nitronaphthalene used in the first step).

[2] The checkers found it convenient to use the mother liquors to recrystallize subsequent batches of glycine salt. Note, however, that no additional amounts of water should be added. As many as 10 crops of glycine salt have been recrystallized from the same solvent without affecting the purity of the product. In this way the yield is increased by about 10%.

[3] The checkers carried out this step satisfactorily using triple quantities.

[4] The precipitate should not be allowed to dry on the filter since it then is difficult to suspend in the next step

III. Properties and Purity of Product

The product gives the correct analytical data for 5-nitronaphthalene-1-sulfonic acid dihydrate and yields a sulfonyl chloride and an amide melting at the correct temperature.[5] It is of suitable purity for use in the L-alanine and L-serine preparation (see p. 9).

IV. Methods of Preparation

5-Nitronaphthalene-1-sulfonic acid has been prepared by the sulfonation of α-nitronaphthalene [6] and by the nitration of naphthalene-1-sulfonyl chloride.[5]

CASEIN

Submitted by MAX S. DUNN, University of California, Los Angeles.
Checked by W. D. CELMER and H. E. CARTER, University of Illinois, Urbana.

I. Principle

Casein is precipitated from skim milk by the addition of 0.5 *N* hydrochloric acid to *p*H 4.8. The precipitate is washed with distilled water to remove soluble chloride and phosphate and with ethanol and ether to remove moisture and fat. The purified product is allowed to stand in air to remove ether and attain moisture equilibrium.

II. Procedure [1]

In a 12-l. round-bottomed flask are placed 1.4 l. of skimmed milk [2] and 8.5 l. of distilled water. The mixture is stirred vig-

[5] H. Erdman and C. Suvern, *Ann.*, 275, 230 (1893).

[6] D. G. Doherty, W. H. Stein, and M. Bergmann, *J. Biol. Chem.*, 135, 487 (1940), W. H. Stein, S. Moore, G. Stamm, C. Chou, and M. Bergmann, *J. Biol. Chem.*, 143, 121 (1942).

CASEIN

[1] Great care should be taken in all manipulations to avoid adding foreign material which, if insoluble in wash solvents, would contaminate the final product. Precipitates should not be allowed to dry until after the final washing with ether since moisture-containing casein dries to a hard difficultly soluble product.

[2] Prepared by centrifuging fresh whole milk.

orously, and 0.5 *N* hydrochloric acid is added slowly (30 minutes) to *p*H 4.8 (about 150 ml. are required; by adding the acid slowly it is possible to tell when proper *p*H is reached by observing the suspension). The mixture is stirred for 10 minutes, the suspension is allowed to settle for 1 hour, and the supernatant liquid is siphoned off.

The residues from three or four such preparations are transferred to a 12-l. round-bottomed flask, distilled water is added to a volume of about 10 l., and the mixture is stirred vigorously for 5 minutes. The suspension is allowed to settle, and the supernatant liquid is siphoned off. The washing operation is repeated three times (a total of 4 washings).

The residues from six or seven precipitations are combined and filtered on a 25-cm. Büchner funnel fitted with two layers of smooth filter paper and an upper layer of hard, lintless filter paper. Suction is applied until the bulk of the water has been removed.[3] The moist residue is transferred to a 5-l. round-bottomed flask, 2.5 l. of distilled water are added, the mixture is stirred vigorously for 5 minutes, and the suspension is filtered as described above. This washing process is repeated twice or until the filtrate gives no test for chloride ion. The residue is suspended in 2 l. of 95% ethanol, the mixture is stirred vigorously for 5 minutes, and the suspension is filtered as described above. This washing process is repeated twice. The residue is suspended in 2 l. of absolute ethanol, the mixture is stirred vigorously for 5 minutes, and the suspension is filtered as described. This washing process is repeated two more times. The residue is suspended in 2 l. of ether, the mixture is stirred vigorously for 5 minutes, and the suspension is filtered as described above. This washing process is repeated, and the purified casein is allowed to stand overnight in large covered evaporating dishes to remove ether and to attain moisture equilibrium. The product is a white, free-flowing powder. The yield of purified casein from seven precipitations is 198 gm.

[3] Do not allow to dry completely. The submitter found that 2 hours were required. The checkers found 30 minutes to be adequate on a hot summer day. At the end of 2 hours the product was dry and hard and was not suitable for use in subsequent steps.

III. Properties and Purity of Product

The product contains 6.2% moisture, 0.55% ash, 0.59% phosphorus, and 14.5% nitrogen.

IV. Methods of Preparation

The preparation of casein by adding dilute acetic acid to skim milk, dissolving the precipitate in dilute alkali, and acidifying the solution with acetic acid was first described by Hammarsten.[4] Van Slyke and Bosworth [5] modified the Hammarsten procedure by dissolving the precipitated casein in ammonium hydroxide and precipitating calcium as calcium oxalate. Van Slyke and Baker [6] precipitated casein with lactic acid or a mixture of hydrochloric and acetic acids and centrifuged an aqueous suspension of the precipitate to remove electrolytes. Cohn and Hendry [7] precipitated casein with a mixture of hydrochloric and citric acids, redissolved the precipitate by adding hydrochloric acid to *p*H 3.0, and reprecipitated the casein by adding base to *p*H 4.6. Essentially this procedure has been employed by other workers.[8] Van Slyke and Carpenter [9] prepared an electrolyte-free product by electrodialyzing casein suspensions.

[4] Hammarsten, *Upsala Läkarefören. Forh*, 9, 363 (1873–1874); quoted by Hammarsten and Hedin, *Textbook of Physiological Chemistry*, 7th ed, trans. by Mandel, John Wiley & Sons, New York, 1914.

[5] L. L. Van Slyke and A. W. Bosworth, *J. Biol. Chem.*, 14, 203 (1913).

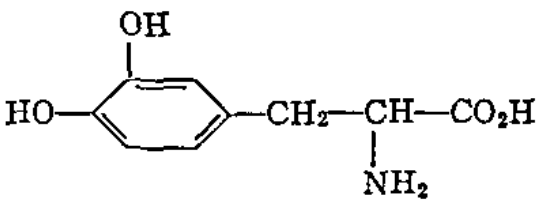
[6] L. L. Van Slyke and J. C. Baker, *J. Biol. Chem.*, 35, 127 (1918).

[7] E. J. Cohn and J. L. Hendry, *Org. Syntheses* Coll. Vol. 2, 120 (1943).

[8] W. M. Clark, H. F. Zoller, A. O. Dahlberg, and A. C. Weimar, *Ind. Eng. Chem.*, 12, 1163 (1920); H. F. Zoller, *Ind. Eng Chem.*, 13, 510 (1921); J. H. Northrup, *J. Gen. Physiol.*, 5, 749 (1923).

[9] Van Slyke and Carpenter, quoted by Bogue, *The Theory and Application of Colloidal Behavior*, McGraw-Hill Book Company, New York, 2, 788 (1924).

β-3,4-DIHYDROXYPHENYL-L-ALANINE



Mol. wt 197.19 ($C_9H_{11}NO_4$)

Submitted by ROBERT R. SEALOCK, Iowa State College, Ames
Checked by HARRIET NEVILLE and H. E CARTER, University of Illinois, Urbana.

I. Principle

Finely ground velvet bean meal (*Vicia fabia*) is extracted with acidified water, and from the extract the proteins are precipitated by the addition of excess lead acetate. From the filtrate, the lead salt of the amino acid is precipitated by making the solution slightly alkaline. The amino acid is obtained by decomposing the lead salt with hydrogen sulfide.

II. Starting Material

Velvet beans may be obtained from southern seed dealers, for example, H. G. Hastings Company, Atlanta, Georgia, or H. M. Franklin and Company, Tennille, Georgia. The germination quality and variety are unimportant. The beans are quite hard but may be successfully ground in the usual feed-store power grinder.

III. Procedure

Two kilograms of finely ground velvet beans contained in a 5-gal. stone crock are extracted overnight (16–18 hours) with 5 l. of 1% acetic acid (50 ml. of glacial acid diluted to 5 l.) and 50 ml. of strong sulfurous acid solution (concentration not critical; prepared by bubbling sulfur dioxide into water). The

mixture is stirred (wooden paddle) occasionally during the first hour or two. The slightly reddish solution is filtered through a circle of heavy canvas cloth on a 10-in. Büchner funnel. The well-pressed residue is washed once with 1 l. of 1% acetic acid.

The residue is twice re-extracted overnight using 3.0 l. of 1% acetic acid and 30 ml. of sulfurous acid each time. The thoroughly washed and pressed residue is then discarded.

To each portion of the filtrate as it is obtained are slowly added 200 ml. of 20% lead acetate solution for each liter of extract, which need not be entirely clear. The mixture is then gently stirred, and a small portion is filtered and tested with a few drops of lead acetate to ensure the presence of excess lead. Different lots of beans may require slightly different amounts of lead acetate. It is usually convenient to allow the protein precipitate to settle overnight. The supernatant is then siphoned off and filtered with suction through an 18.5-cm. No. 1 Whatman paper, the precipitate being finally transferred to the funnel.

To the filtrate, 10% ammonium hydroxide is added slowly with gentle stirring until the solution is *p*H 7.0–7.5 (Nitrazine paper), care being taken to ensure an excess of lead acetate at all times. After the lead salt of the amino acid has settled out, the supernatant is siphoned off and discarded, and the precipitate is collected with suction on an 18.5-cm. paper. The precipitate may be purified by resolution in acetic acid as above or by thorough suspension in approximately 10 volumes of 1% lead acetate at *p*H 7.0–7.5. However, for most purposes this procedure has not been found necessary.

To remove the lead, the moist salt [1] from 2 kg. of beans is finely suspended [2] in 15 l. of water and treated with hydrogen sulfide with rapid stirring until the lead sulfide flocculates. The lead sulfide is allowed to settle; the supernatant and, finally, the precipitate are filtered with suction. The filtrate is concentrated to one-tenth volume in vacuum with nitrogen or carbon dioxide

[1] The dry salt is difficult to suspend and decompose. Consequently, it should be kept in the moist form even when not being treated immediately.

[2] The Waring blendor is ideal for this purpose, small portions of water being used. The balance of the water is then added to the suspended salt in the reaction flask.

gas admitted through the capillary. As the solution becomes more concentrated it is necessary to reduce the bath temperature to 50–60°. A few drops of sulfurous acid are added after complete removal of hydrogen sulfide, and the stoppered solution, containing amino acid crystals, is left in the icebox for 2–3 days. The crystalline product is collected with suction and washed thoroughly with 3 portions of cold water. Drying is accomplished immediately by washing with 3 portions of 95% ethanol and 2 portions of ether. The combined filtrate and water washings are again concentrated, and a second crop of crystals is obtained, the same precautions against oxidation being taken. The yield of crude material is 47–50 gm. (approximately 90% of that present in the beans originally).

For purposes of recrystallization the crude material is suspended in 40 volumes of water. A few drops of sulfurous acid and 20 gm. of hydrochloric acid-washed Norite are added, and the mixture is digested at the boiling point for 20 minutes. After filtration a few more drops of sulfurous acid are added and the solution is stored in the cold for 2–3 days. The white crystalline material is separated, washed, and dried as previously described. Concentration of the mother liquor yields an addition 1–2 gm., making the total yield 45–48 gm. The thoroughly dried material when stored in a tightly stoppered container out of the light remains colorless for several years.

IV. Purity and Properties of Product

The recrystallized compound melts with decomposition at 284–286° when the capillary is placed in a bath at 260°. A 1% solution in 4% hydrochloric acid gives $[\alpha]_D = -12.0°$. At 20° it dissolves in 200 parts of water, and at boiling temperature in 40 parts of water. Arnow's method of colorimetric analysis [3] has been used with various types of biochemical samples with satisfactory results. β-3,4-Dihydroxyphenyl-L-alanine in solution is rapidly oxidized by atmospheric oxygen.

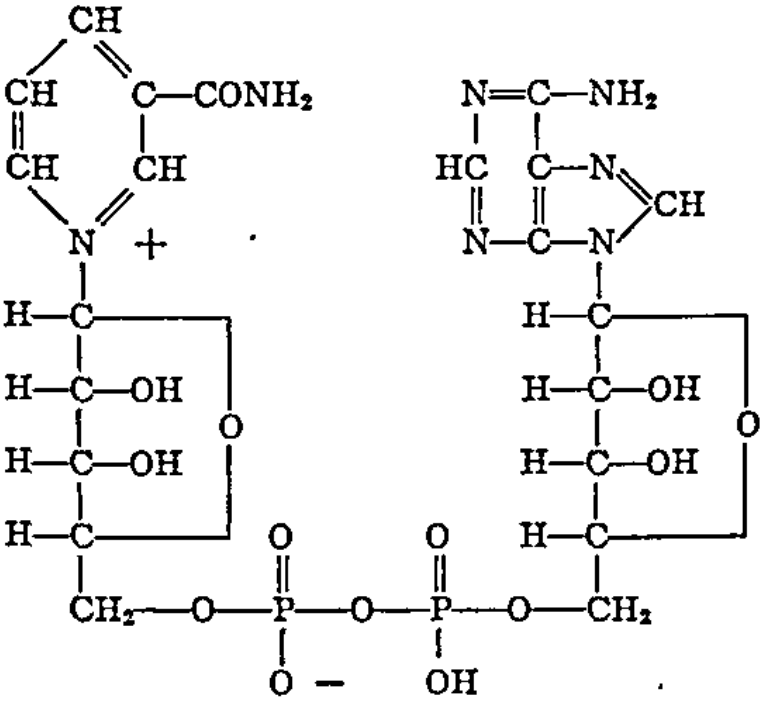
[3] L. E. Arnow, *J. Biol Chem.*, 118, 531 (1937).

V. Methods of Preparation

The present procedure is an adaptation of the methods of Miller,[4] Guggenheim,[5] and Torquati.[6] The natural isomer has also been prepared by resolution of the synthetic acetyl amino acid [7] and from L-tyrosine by nitration and subsequent conversion.[8]

DIPHOSPHOPYRIDINE NUCLEOTIDE

(DPN; Cozymase)



Mol. wt. 663.3 ($C_{21}H_{27}O_{14}N_7P_2$)

Submitted by G. A. LEPAGE, University of Wisconsin, Madison.
Checked by ROBERT K. CRANE and ERIC BALL, Harvard University Medical School, Boston.

I. Principle

The DPN present in yeast is extracted by mixing into hot water. The killed yeast is filtered, and the filtrate is treated with

[4] E. R. Miller, *J. Biol. Chem.*, 44, 481 (1920).
[5] M. Guggenheim, *Z. physiol. Chem*, 88, 276 (1913).
[6] T. Torquati, *Arch. farm. sper.*, 15, 213, 308 (1913).
[7] C. R. Harington and S. S. Randall, *Biochem. J.*, 25, 1028 (1931).
[8] E. Waser and M. Lewandowski, *Helv. Chim. Acta*, 4, 657 (1921).

basic lead acetate which precipitates phosphate esters, proteins, and other material. DPN is precipitated from the supernatant fluid as the silver salt. The salt is decomposed with hydrogen sulfide, and the DPN is purified by a lead-ethanol fractionation and by absorption on charcoal and elution with aqueous pyridine.

II. Starting Material

Baker's yeast appears to be the best source of DPN. If several sources are available locally a survey should be made to determine which yeast is best since there is considerable variation in quality. It is essential that the yeast be fresh. Storage for 1 week in the refrigerator (0–3°) reduces the yield of DPN by about 50%.

III. Procedure

Four liters of water are heated to 92° in a 12-l. flask. Crumbled, pressed yeast (10 lb.) is added as rapidly as possible, the temperature being maintained at 90–92°. The mixture is stirred vigorously. When the addition is completed (8–15 minutes) the extraction mixture is cooled to room temperature rapidly in a cold-water bath. The yeast suspension is filtered on large Büchner funnels (25-cm.) with suction, either with a filter aid (100 gm. of Super-Cel per liter) or, preferably, through a pressure filtering device.[1] The filtrate (4–4.3 l.) is treated with one-tenth its volume of 25% basic lead acetate ($Pb(OAc)_2 \cdot Pb(OH)_2$), and the precipitate is filtered on large Büchner funnels using a filter aid or with a pressure filter. The filtrate is adjusted to *p*H 6.5 with acetic acid (very little is required). Fifty milliliters of 25% silver nitrate solution are added with stirring, and the resulting precipitate is permitted to settle in the refrigerator. The bulk of the supernatant fluid is decanted, and the remainder is separated by centrifugation preferably in the cold. The precipitate is washed in the centrifuge bottles with 3 successive portions of cold distilled water, each approximately 3 times the volume of the

[1] A horizontal pressure filter of laboratory scale suitable for this operation is manufactured by the Sparkler Filter Company, Mundelein, Illinois. It can be operated from laboratory air-pressure lines or with a pump and motor

packed precipitate (about 150 ml. for each washing). The washed precipitate is suspended in 50–70 ml. of water and decomposed with hydrogen sulfide. The silver sulfide is removed with centrifugation and is washed with a small volume of water and discarded. The combined supernatant and washings are aerated free of hydrogen sulfide. Five volumes of cold acetone are added, and the precipitate is centrifuged in the cold and dried in vacuum over sulfuric acid. The yield of crude product assaying 30% DPN is 1.2–1.5 gm.[2]

To a solution of 10 gm. of crude DPN in 775 ml. of 0.1 *M* acetic acid are added 25 ml. of 25% lead acetate solution ($Pb(OAc)_2$) and 2 l. of 95% ethanol. The mixture is cooled to 0°, and the precipitate is centrifuged and discarded. The supernatant fluid is treated with 50 ml. of 25% silver nitrate solution (excess). The silver salt of DPN is centrifuged in the cold and washed once with 2 volumes of cold water (about 100 ml.) containing a little silver nitrate. The silver salt is suspended in 100–150 ml. of water and decomposed with hydrogen sulfide. The silver sulfide is centrifuged, and the hydrogen sulfide is removed from the supernatant solution by aeration.[3] The solution is neutralized to *p*H 7 with sodium hydroxide[4] and diluted to 400 ml. with distilled water. Seventy-two grams of acid-washed, air-dried Norite A decolorizing charcoal are added to the solution, and the mixture is stirred vigorously for 20 minutes and filtered through a fritted-glass funnel (fine porosity). The filtrate contains no DPN and is discarded. The charcoal is washed on the filter with 300 ml. of 2% trichloroacetic acid (removes sodium ions) and with 100 ml. of water. No DPN is lost at this step. The washed charcoal is suspended in 360 ml. of a 20% solution (by

[2] This amount of material can be carried through the subsequent steps with good recoveries. However, it is convenient to combine several batches at this stage and carry 10 gm. or more through the remaining procedure at one time.

[3] DPN of 55–57% purity is obtained with 90% recovery by adding acetone to this solution. Such preparations, however, when tested in biological systems exhibit properties attributable to traces of heavy metals. These effects are also found with the 30% DPN preparations.

[4] Caution should be exercised to avoid local areas of high *p*H during the neutralization. Use a solution not more than 0.5 *N*.

volume) of purified pyridine [5] in water, and the suspension is stirred vigorously for 20 minutes. The charcoal is filtered on the fritted-glass funnel, and the elution is repeated with a second 360-ml. portion of 20% pyridine solution. The combined eluates are concentrated under reduced pressure at 30–35° (bath temperature). The pyridine prevents the *p*H from decreasing unduly. When the volume has been reduced to 40–50 ml. (*p*H now 4.0–4.5) the solution is filtered to remove the last traces of charcoal. The DPN is precipitated from the filtrate with 6–7 volumes of acetone in the cold. This operation is best carried out with small successive portions in a 50-ml. centrifuge tube.[6] In this way loss of material adhering to the sides of the vessel is minimized. After the precipitation is completed the product is dried in vacuum over sulfuric acid. It then can be readily scraped from the tube and ground in a mortar. The powder is placed in an appropriate container and dried again in vacuum before sealing, since the material is very hygroscopic. Eighty per cent of the crude starting material is recovered in the final product, which assays 63–73% DPN. This material is free of trace metal effects.

IV. Properties and Purity of Product

Chemical analyses are of limited use in evaluating the purity of DPN preparations since the impurities resemble DPN in composition. Most, if not all, of the impurity consists of myoadenylic acid. Some water (3–5%) also remains. Adenylic acid is innocuous for most of the reactions in which DPN is used. Also it has been pointed out [7] that in a biological system there is always a possibility of decomposition of DPN to yield adenylic acid or its precursors. Material obtained by this preparation is, therefore, satisfactory for most purposes.

In assaying DPN preparations of high purity the most precise

[5] The colored impurities present even in reagent-grade pyridine tend to accumulate in the final product. Pyridine redistilled from glass and protected from light is satisfactory.

[6] Purified DPN is more difficult to centrifuge than the crude preparations. The DPN precipitates as the free acid.

[7] F. Schlenk and T. Schlenk, *Arch. Biochem.*, 14, 131 (1947)

method is the measurement of absorption at 340 mμ after reduction of the sample with hydrosulfite in alkaline solution.[8] If a standard of known purity is available DPN may be assayed in biological systems.[9] These have the advantage of being specific and of detecting toxic impurities, such as traces of heavy metals. The submitter made use of the facilities of Dr. V. R. Potter to have assays carried out using the malic dehydrogenase system of animal tissues, which requires DPN.[10] Final preparations were also assayed by the Beckman spectrophotometer.[8c]

DPN titrates as a monobasic acid. As the free acid it is stable in solution for a week or more at 0°; when neutralized it is stable for 2 weeks at 0°. Boiling of neutral solutions causes gradual decomposition. Boiling of acid solutions (*p*H 2) results in complete destruction in 20–30 minutes. The free acid is very soluble. High-purity DPN is soluble to the extent of 0.5 mg. per ml. in dilute silver nitrate at 0°. In crude materials, such as yeast extracts, the solubility of the silver salt is much less. The lead salt of DPN has a solubility (*p*H 5) exceeding 1 mg. per ml.

V. Methods of Preparation

Cozymase has been prepared from yeast,[8c,11] from rabbit muscle,[12] and from erythrocytes.[8a]

In most of the procedures a preliminary separation is effected by precipitation of the silver salt of DPN. Further purification may be achieved by precipitation with phosphotungstic acid [11a,12] and cuprous chloride [11a,b,d,12] by the use of alumina columns,[11b] and by the adsorption of DPN on charcoal followed by elution

[8] (*a*) O. Warburg and W. Christian, *Biochem. Z.*, 287, 291 (1936); (*b*) H. v. Euler, E. Adler, and H. Hellström, *Z. physiol. Chem.*, 241, 239 (1936); (*c*) G. A. LePage, *J. Biol. Chem.*, 168, 623 (1947). (Cf. also Schlenk, *A Symposium on Respiratory Enzymes*, p 104, University of Wisconsin Press, 1941.)

[9] B. J Jandorf, F. W. Klemperer, and A. B. Hastings, *J. Biol. Chem.*, 138, 311 (1941).

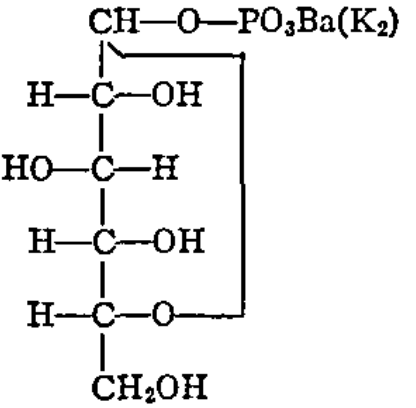
[10] V. R. Potter, *J. Biol. Chem.*, 165, 311 (1946).

[11] (*a*) [illegible] v. Euler and F. Schlenk, *Z* [illegible] ıd D. E. Green, *J. Biol. C* [illegible], 138, 305 (1941).

[12] S. Ochoa, *Biochem. Z.*, 292, 68 (1937).

with amyl alcohol [11d] or aqueous pyridine.[8c] A mixture of alcohol, ether, and sulfuric acid has been used to extract DPN from yeast with very satisfactory results.[13] The present method is that of LePage.[8c]

THE α-GLUCOSE-1-PHOSPHATES



$C_6H_{11}O_8PO_4Ba \cdot 3H_2O$ (Mol. wt. 449.54)
$C_6H_{11}O_8PO_4K_2 \cdot 2H_2O$ (Mol. wt. 372 35)

Submitted by M. E. KRAHL and C F. CORI, Washington University School of Medicine, St. Louis
Checked by CORNELIUS F. STRITTMATTER IV and ERIC BALL, Harvard Medical School, Boston

I. Principle

Glucose $\xrightarrow{(CH_3CO)_2O}$ β-glucose pentaacetate $\xrightarrow[CH_3CO_2H]{HBr}$ α-Tetraacetyl glucose-1-bromide $\xrightarrow{Ag_2PO_4}$ tri-(tetraacetylglucose-1)-phosphate $\xrightarrow[CH_3OH]{HCl}$ α-glucose-1-phosphoric acid + undetermined products

α-Glucose-1-phosphoric acid $\xrightarrow{Ba(OH)_2}$ α-barium glucose-1-phosphate $\xrightarrow{K_2SO_4}$ α-dipotassium glucose-1-phosphate

II. Starting Material

A. β-Glucose pentaacetate.[1] Two hundred grams of dry, finely powdered crystalline glucose, 100 gm. of finely powdered

[13] J. B. Sumner, P. S. Krishman, and E. B. Sisler, *Arch. Biochem*, **12**, 19 (1947).

THE α-GLUCOSE-1-PHOSPHATES

[1] An alternative procedure is described in *Org. Syntheses*, **22**, 1 (1942).

anhydrous sodium acetate, and 1 kg. of reagent-grade acetic anhydride are placed successively in a 3- to 4-l. flask. The mixture is brought to the temperature of a boiling water or steam bath and agitated vigorously until all the suspended material dissolves or until only a small residue of undissolved sodium acetate remains (0.5–1.5 hours). The agitation and heating are then continued for an additional 2 hours. The reaction mixture is cooled with tap water and poured, with motor stirring, over 4 l. of crushed ice. A white, semicrystalline mass is deposited; the stirring is continued until the ice melts.

The precipitate of glucosepentaacetate is collected on a Büchner funnel, and all possible water is pressed out of the spongy mass. The precipitate is then resuspended in 4 l. of water, stirred 2 hours, and left, without stirring, at 5° overnight in order to extract and decompose acetic anhydride. The glucose pentaacetate is again filtered, and all possible water is pressed out. The crude material is dissolved in 1 l. of boiling 95% ethanol, and the solution is filtered and cooled to room temperature or below. The crystalline precipitate is filtered by suction and again recrystallized from 1 l. of hot 95% ethanol, giving white crystals of β-glucose pentaacetate. These are collected by suction and dried over calcium chloride (m.p. 124–126° [cor.]; yield, 303–325 gm., 70–75%). This product is satisfactory for use in the next step although the melting point is lower than that of the pure material (132°).

B. α-Tetraacetyl glucose-1-bromide.[1,2] One hundred and seventy-five milliliters of a 30% solution of anhydrous hydrobromic acid in glacial acetic acid (Eastman Kodak Company) are placed in a 500-ml. glass-stoppered Erlenmeyer flask and cooled to about 5°. One hundred grams of dry glucose pentaacetate are added, and the flask is restoppered and shaken gently until a clear solution (light yellow) is obtained. The flask and contents are then warmed to room temperature and allowed to stand at room temperature for 3 hours.

The reaction mixture is poured slowly, with vigorous motor stirring, over 1.7 kg. of ice. The stirring is continued until the

[1] D. Freudenberg, A. Noë, and E. Knopf, *Ber.*, 60, 238 (1927).

ice melts, with interruptions every 5–10 minutes in order to break up with a stirring rod any lumps which may occlude substantial quantities of the undiluted reaction mixture.

The semicrystalline mass of crude tetraacetyl glucose-1-bromide is collected on a Büchner funnel and washed with 300–500 ml. of ice water, and all possible water is pressed out. The product is not stable at this stage and must be immediately recrystallized. It is dissolved in 800 ml. of 99–100% methanol at room temperature or below [3] and separated from undissolved impurities by filtration. The clear, colorless solution is chilled in an ice bath; an equal volume of ice water is then added slowly, with vigorous stirring. The crystalline tetraacetyl glucose-1-bromide is filtered and washed with 100 ml. of ice water, and all possible water is pressed out.

The recrystallization is repeated, and the pure white product is pressed out on a porous plate to remove part of the solvent, then dried as rapidly as possible over phosphorus pentoxide in vacuum (m.p. 89–90° [cor.]; yield 85–95 gm., 80–90%). This product is stable for months if kept over calcium chloride or phosphorus pentoxide.

C. Trisilver phosphate.[4] It is desirable that the preparation and handling of this material be carried out under reduced illumination; the light from an ordinary photographic dark-room lamp suffices. Eleven and nine-tenths grams of disodium phosphate (Na_2HPO_4) (0.084 mole) and 42.7 gm. of silver nitrate (0.252 mole) are each dissolved in 1 l. of water; the solution of the former is poured slowly into that of the latter, giving a flocculent, canary-yellow precipitate. The mixture is stirred mechanically for 15–30 minutes. The precipitate is collected on a Büchner funnel and washed successively with 500 ml. of water, 200 ml. of absolute ethanol, and 200 ml. of commercial anhydrous ether. Air is drawn through the mass for 30 minutes (yield, 18 gm.,

[3] Reaction of the bromide with methyl alcohol to form tetraacetyl methyl-glucoside is minimized by carrying out this recrystallization step as rapidly as possible and at the lowest temperature at which the crude moist product is fully soluble in the methanol.

[4] C. F. Cori, S. P. Colowick, and G. T. Cori, *J. Biol. Chem*, 121, 465 (1937).

50%[5]). The dry, granular, yellow product should be used on the same day it is made, as it tends to darken when stored.

Trisilver phosphate may also be prepared by the method of Lipmann and Tuttle.[6] A solution of 103 gm. (0.73 mole) of disodium phosphate (Na_2HPO_4) in 600 ml. of water is heated to boiling, and to it is added slowly a solution of 50 gm. (0.294 mole) of silver nitrate in 250 ml. of water. The reaction mixture is boiled for a few minutes and then cooled to room temperature. The precipitate settles rapidly. The supernatant fluid is decanted off, and the precipitate is washed by decantation 5 successive times, at hourly intervals, using about 5 l. of water each time.[7] The precipitate is collected on a Büchner funnel, carefully separated from the filter paper, and dried overnight at 130°. It is then ground in an agate mortar and stored over calcium chloride in the dark. Under these conditions the product is stable and may be employed when needed (yield, 40 gm., 98% of theoretical amount from 50 gm. of silver nitrate).

III. Procedure

A. Condensation of tetraacetyl glucose-1-bromide with trisilver phosphate. It is advisable that this step be carried out under the same conditions of reduced illumination as specified above for the preparation of the trisilver phosphate. Seventeen and five-tenths grams of trisilver phosphate (0.042 mole), 50 gm. of tetraacetyl glucose-1-bromide (0.122 mole), and 150 ml. dry benzene[8] are placed in a three-necked 500-ml. flask fitted with a mercury-sealed stirrer and a reflux condenser equipped with a calcium chloride tube. The mixture is stirred vigorously to

[5] When equimolecular quantities of disodium phosphate and silver nitrate are mixed, the reaction is very complex and results in an incomplete yield of trisilver phosphate. (See J. W. Mellor, *A Comprehensive Treatise on Inorganic and Theoretical Chemistry*, Vol. III, p. 485, Longmans, Green and Company, New York, 1923)

[6] F. Lipmann and L. C. Tuttle, *J. Biol. Chem.*, 153, 571 (1944).

[7] This washing is not necessary but helps to reduce the amount of mono- and disilver phosphate occluded by the main precipitate of trisilver phosphate.

[8] J. F. Norris, *Org. Syntheses* Coll. Vol. 1, 549, 2nd edition, 1941.

dissolve the bromide and disperse the silver phosphate. The benzene solution is then refluxed for 1 hour.[9] As the reaction proceeds, the yellow trisilver phosphate is replaced by cream-colored silver bromide.

The mixture is transferred to four 50-ml. centrifuge tubes, which are capped to protect the product from moisture, and centrifuged until the supernatant solution is clear. This supernatant solution is decanted into a 1-l. suction flask, and the silver salts are discarded without washing.[10]

The suction flask containing the benzene solution of the reaction product is closed with a rubber stopper and attached to a water pump. The benzene is distilled under reduced pressure, the contents of the flask being kept at 30–40° by occasional warming in a water bath, until the mixture is reduced to a very thick syrup. The suction flask is then attached to a Hyvac pump and evacuated until the residual syrup is converted to a pure white glassy product which fills the flask in the form of a froth; this usually requires 2–3 hours, but pumping may be continued overnight or longer, if desired (yield, 40 gm., 91%, assuming a molecular weight of 1088 for the condensation product). According to the method of analysis given in the next section, the product appears to be a mixture containing tri-(tetraacetyl glucose-1)-phosphate, di-(tetraacetyl glucose-1)-phosphate, a small amount of tetraacetyl glucose-1-phosphate, and some reducing material derived from the tetraacetyl glucose-1-bromide which did not condense with the trisilver phosphate.

B. Methods for characterizing the condensation product of tetraacetyl glucose-1-phosphate and trisilver phosphate and for following the hydrolysis in step C. 1. *Total phosphate analysis.* The condensation product is dissolved in 98–100% methanol to give a clear solution containing 1.0 mg. per ml. (about 25 μg. of

[9] The degree of condensation is less at 30 minutes than at 1 hour but tends to decrease progressively after 1 hour, owing probably to hydrolytic splitting of the product as the refluxing is continued.

[10] A small amount of condensation product may be recovered by washing the silver salts with a 50- to 100-ml. portion of dry benzene, but, after centrifuging, the wash fluid frequently contains colloidally dispersed silver salts which tend to interfere with the next step in the synthesis.

phosphorus per ml.). A 1.0-ml. aliquot is placed in a large Pyrex test tube, 1.0 ml. of 5 *N* sulfuric acid is added, and the tube is heated over a microburner until white fumes appear. One drop of 10% hydrogen peroxide is added, and the tube is heated again until a water-clear solution is obtained. Three milliliters of water are added, and the solution is brought to a boil to decompose pyrophosphate. The phosphorus is then determined colorimetrically by the Fiske and Subbarow method.[11]

One milligram of tri-(tetraacetyl glucose-1)-phosphate would contain 28.4 μg. of phosphorus. The condensation product obtained by the methods described above usually contains approximately 85–95% of this theoretical amount.

2. *Titration of primary and secondary phosphate groups.* The number of equivalents of primary and secondary phosphate groups may be estimated with an accuracy of about ±5–10% as follows: A sample of the condensation product is dissolved in 98–100% methanol. An aliquot of 0.2–0.8 ml. containing approximately 1×10^{-5} mole of phosphate is transferred to a 10- to 20-ml. beaker or cup mounted to receive a glass electrode and a calomel electrode for titration. Enough methanol and water are added by pipette to give 4.0 ml. of a solution 20% by volume in methanol.[12] This solution is titrated with 0.01 *N* sodium hydroxide as the *p*H values are read on a *p*H meter.[13]

By titration of known solutions containing 0.005 *M* phosphoric acid in 20% methanol solution, it has been observed that the end points for titration of primary and secondary phosphate groups are *p*H 4.5 and *p*H 8.2 respectively. Hence, for each 1×10^{-5} equivalent of total phosphorus, the number of equivalents of primary phosphate equals the number of milliliters of 0.01 *N* NaOH required to bring the solution up to *p*H 4.5,[14] the

[11] C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, 66, 375 (1925).

[12] The condensation product is not completely soluble in this volume at concentrations of methanol less than 20% by volume.

[13] For the observations here described, a Leeds and Northrup *p*H assembly No. 7661-A1, equipped with a 10-ml. titration vessel, was used.

[14] When this titration is carried out upon the solution of the condensation product in 0.2 *N* hydrochloric acid in methanol, the volume of 0.01 *N* sodium hydroxide required to bring the same volume of the parent solution containing 0.2 *N* hydro-

number of equivalents of secondary phosphate equals the number of milliliters of 0.01 *N* sodium hydroxide required to take the solution from *p*H 4.5 to *p*H 8.2 (a blank equal to the number of milliliters of the 0.01 *N* sodium hydroxide required to titrate an equivalent volume of 20% methanol-water from *p*H 4.5 to *p*H 8.2 is subtracted from this value).

If the condensation of tetraacetyl glucose-1-phosphate proceeded to completion, the condensation product would contain no titratable primary or secondary phosphate groups. The condensation product actually contains a variable quantity of both primary and secondary phosphate groups; for example, one sample showed by titration about 0.5 equivalent of free primary phosphate and about 0.2 equivalent of free secondary phosphate per mole of total phosphate. Since no inorganic phosphate was present (see below), there were in this sample 0.2 mole of tetraacetyl glucose-1-phosphate, 0.3 mole of di-(tetraacetyl glucose-1)-phosphate, and 0.5 mole of tri-(tetraacetyl glucose-1)-phosphate per mole of total phosphate.

3. *Inorganic phosphate.* An aliquot of the condensation product containing about 25–100 μg. of total phosphorus in 0.1–0.2 ml. of methanol is diluted with 1 ml. of water and analyzed for inorganic phosphate by the method of Lowry and Lopez;[15] this method is suggested because it minimizes the chance of hydrolysis of the acid-labile α-glucose-1-phosphate.

The condensation product contains no inorganic phosphate when analyzed either by this method or by that of Fiske and Subbarow.[11]

C. Deacetylation and hydrolysis of the condensation product from IIIA to obtain α-glucose-1-phosphoric acid. The following procedure has given consistently satisfactory results. However, the method is empirical, and the optimum time for stopping the hydrolysis varies from batch to batch; this optimum time must be determined in each instance by following the liberation of

chloric acid in methanol (before solution of the condensation product) to *p*H 4.5 must be subtracted from the total volume of 0.01 *N* sodium hydroxide required to reach *p*H 4.5.

[15] O. H. Lowry and J. A. Lopez, *J. Biol Chem.*, 162, 421 (1946).

inorganic phosphate; concurrent determination of primary and secondary phosphate groups may also prove helpful in obtaining optimum yields.

Forty grams of the tri-(tetraacetyl glucose-1)-phosphate product from IIIA are mixed with 800 ml. of approximately 0.2 *N* methanolic hydrochloric acid (14.0 ml. of concentrated hydrochloric acid (sp. gr. 1.19) diluted to 850 ml. with absolute methanol) in a 1-l. Erlenmeyer flask. The flask is stoppered, and the clear, colorless solution is allowed to stand at 20–25°. At hourly intervals, 0.01- to 0.02-ml. aliquots are withdrawn and analyzed for inorganic phosphate as described above. If possible, 0.2-ml. aliquots are also withdrawn at hourly intervals and titrated for primary and secondary phosphate groups as described above.

When the inorganic phosphate has risen to 20% of the total phosphate, the reaction mixture is diluted with an equal volume of methanol and brought to *p*H 8.0–8.5 by the addition of 0.3 *N* barium hydroxide.[16] The further handling of the mixture is described in IIID below.

The course of the appearance of primary, secondary, and inorganic phosphate is illustrated by the data of Table I. The

TABLE I

EQUIVALENTS OF PRIMARY AND SECONDARY PHOSPHATE AND MOLES OF INORGANIC PHOSPHATE PER MOLE OF TOTAL PHOSPHATE DURING THE HYDROLYSIS OF TRI-(TETRAACETYL GLUCOSE-1)-PHOSPHATE BY 0.2 *N* HYDROCHLORIC ACID IN METHANOL, 20°

Hours	Primary	Secondary	Inorganic	Secondary minus Inorganic
0	0.55	0.07	0.00	0.07
1	0.68	0.24	0.01	0.23
2	0.78	0.28	0.02	0.26
3	0.82	0.34	0.03	0.31
4	0.88	0.38	0.05	0.33
5	0.92	0.41	0.08	0.33
6	0.97	0.50	0.10	0.40
8	0.97	0.76	0.22	0.54
9	0.99	0.82	0.27	0.55
10	1.06	0.80	0.34	0.46
24	1.06	0.99	0.93	0.06

[16] The checkers used thymol blue as the indicator, accepting color changes observed in aqueous solutions as applicable here.

optimum concentration of monosubstituted phosphate (secondary minus inorganic) here occurred after about 8–9 hours at 20°. Other experiments have shown different optimum times (the checkers found 8 hours to be required), so that the inorganic phosphate must be followed and the reaction stopped at about 20–25% inorganic phosphorus.[17] Our experience indicates that at this point the concentration of monosubstituted phosphate is near its maximum and also that a substantial proportion of the monosubstituted product has been deacetylated.[18]

D. Isolation of α-barium glucose-1-phosphate from the reaction mixture of IIIC. After the addition of barium hydroxide as described above, the flask and reaction mixture are left overnight at 0–5° to allow the precipitate of barium glucose-1-phosphate to form and settle.[19] The precipitate is collected by sharp centrifuging, and the supernatant fluid is discarded.

The barium precipitate obtained from the hydrolysis of 40 gm. of condensation product is then extracted at room temperature successively with 60, 40, 40, 40, and 40 ml. of water, shaking vigorously for 10–15 minutes during each extraction, then centrifuging. The supernatant fluids [20] are pooled, and 2 volumes of ethanol are added to reprecipitate the barium salt.

The chilling, centrifuging, and re-extractions are repeated until the precipitate is completely soluble in the first 140 ml. of

[17] Dr. Earl Sutherland obtained the same yield of barium glucose-1-phosphate as obtained by this procedure by carrying out the hydrolysis at 50° for 2 hours, by which time inorganic phosphate had reached 20% of total phosphate.

[18] Deacetylation of the glucose produces mainly methyl acetate, as little or no free acetic acid appears by titration. Titration with known mixtures of phosphate and acetate under the conditions prevailing in the method described above showed that any acetic acid liberated would titrate between *p*H 4 5 and *p*H 6 1.

[19] During this period the *p*H falls, possibly owing to liberation of acetyl groups not previously removed from the tetraacetyl glucose residues during the acid hydrolysis. The *p*H should be brought back to 8 0–8 5 with barium hydroxide before separation of the barium precipitate.

[20] The first extraction serves mainly to remove the alcohol from the packed precipitate and dissolves very little of the barium salt. This method of isolating the barium glucose-1-phosphate apparently depends on the fact that the deacetylated product is more soluble in water than the barium salt of the intermediate products which have not been completely deacetylated The barium salt of inorganic phosphate is practically insoluble in water.

water used for extraction. (Three re-extractions were needed by the checkers.) It is then precipitated a final time; the barium glucose-1-phosphate is collected by centrifuging and dried over phosphorus pentoxide (yield, 3–6 gm., 16–33% of the theoretical yield from tetraacetyl glucose-1-bromide, after allowance for loss of 2 of every 3 tetraacetyl glucose residues during hydrolysis of the intermediate).

Elementary analyses [4] indicate this product to be $C_6H_{11}O_5PO_4Ba \cdot 3H_2O$. Rotation (anhydrous basis), $[\alpha]_D^{25} = +75°$ (1.26% solution in water).

E. Preparation of crystalline dipotassium glucose-1-phosphate. Five grams of the barium salt are dissolved in 100 ml. of water. An exactly equivalent amount of 10% potassium sulfate is added; the precipitate of barium sulfate is centrifuged and discarded. Ethanol (95–100%) is added to the supernatant fluid until a persistent turbidity appears. The sample is left at room temperature for 2 hours or more, during which time crystals of dipotassium glucose-1-phosphate deposit upon the walls of the vessel. More ethanol is added to the point of persistent turbidity, after which further crystals deposit. This intermittent addition of ethanol is continued at intervals of 1 or 2 hours or more until 1.7 volumes of ethanol have been added. The mixture is then chilled for a few hours. The crystals are collected by centrifuging and dried over phosphorus pentoxide (yield, 3–3.5 gm., 73–85%). Elementary analyses [21] indicate this product to be $C_6H_{11}O_5PO_4K_2 \cdot 2H_2O$. Rotation, $[\alpha]_D^{20} = +78°$ and $[\alpha]_{5491}^{20} = +90°$ (4% solution in water).

IV. Properties and Purity of Product

The purity of the synthetic α-glucose-1-phosphate is established by elementary analyses,[4,21] lability to acid hydrolysis,[4,22] degree of conversion to glucose-6-phosphate by phosphoglucomutase,[23] and optical rotation.[4,21] The ester is completely hydrolyzed to glucose and inorganic phosphate by heating at 100° for 10 min-

[21] M. L. Wolfrom and D. E. Pletcher, *J. Am. Chem. Soc.*, 63, 1050 (1941).
[22] W. R. Meagher and W. Z. Hassid, *J. Am. Chem. Soc.*, 68, 2135 (1946).
[23] G. T. Cori, S. P. Colowick, and C. F. Cori, *J. Biol. Chem.*, 124, 543 (1938).

utes in 1 *N* sulfuric acid. The free acid gives $[\alpha]_D^{25} = +120°$ (1% solution in water),[4] and the anhydrous barium salt has an $[\alpha]_D^{25} = +75°$ (1.26% solution in water).[4] The literature contains detailed data on the hydrolysis and dissociation constants of glucose-1-phosphate.[4,22]

α-Glucose-1-phosphate is converted by the enzyme phosphoglucomutase into an equilibrium mixture of 95% glucose-6-phosphate and 5% glucose-1-phosphate.

It is converted to glycogen by phosphorylase [24] and reacts reversibly with fructose to form sucrose under the action of a bacterial phosphorylase.[25] β-Glucose-1-phosphate and the α-isomers of aldose-1-phosphates other than glucose are not attacked by phosphoglucomutase, polysaccharide phosphorylase, or sucrose phosphorylase.

V. Methods of Preparation

A. Synthetic methods. The only synthetic method available for glucose-1-phosphate is that described above, which is essentially the procedure of Cori, Colowick, and Cori.[4] This same method has been applied to the synthesis of galactose-1-phosphate,[26,27] maltose-1-phosphate,[23] mannose-1-phosphate,[26] and xylose-1-phosphate.[22]

β-Glucose-1-phosphate has been synthesized with silver dibenzyl phosphate as the phosphorylating agent [28] and also with "monosilver" phosphate as the phosphorylating agent.[29]

B. Enzymatic phosphorolysis of glycogen or starch. Various methods have been described for the preparation of α-glucose-1-phosphate by the action of muscle phosphorylase on glycogen [4,30]

[24] For a summary of the literature on this reaction see G. T. Cori, M. A. Swanson, and C. F. Cori, *Federation Proc*, 4, 234 (1945).

[25] W. Z. Hassid, M. Doudoroff, and H. A. Barker, *J. Am. Chem. Soc*, 66, 1416 (1944).

[26] S. P. Colowick, *J. Biol. Chem*, 124, 557 (1938)

[27] H. W. Kosterlitz, *Biochem J*, 33, 1087 (1939).

[28] M. L. Wolfrom, C. S. Smith, D. E. Pletcher, and A E. Brown, *J. Am. Chem. Soc.*, 64, 23 (1942).

[29] F. J. Reithel, *J. Am Chem. Soc.*, 67, 1056 (1945).

[30] W. Kiessling, *Biochem Z.*, 298, 421 (1938).

or starch [31] and by the action of phosphorylase from pea seeds [32] or potatoes [33, 34, 35] upon starch according to the reaction: Glycogen (or starch) + phosphate $\rightleftarrows$ α-glucose-1-phosphate. The enzymatic equilibrium at *p*H 6.5 corresponds to a ratio of 80 parts of orthophosphate to 20 parts of glucose-1-phosphate. The phosphorylase preparations must be sufficiently purified to ensure the absence of phosphoglucomutase. Other interfering enzymes in crude phosphorylases are amylases and phosphatases.

The α-glucose-1-phosphate prepared from natural sources, in contrast to synthetic samples, usually contains polysaccharide impurities which activate muscle [24] and potato [36] phosphorylase. Ion exchange resins [37] and Carboraffin C [38] have been used to remove these impurities, thereby making possible the preparation of highly purified α-glucose-1-phosphate of natural origin.

The identity of the natural and synthetic isomers has been established by a variety of chemical and enzymatic data.[4, 22, 30]

L-GLUTAMINE

$$COOH \cdot CH(NH_2) \cdot CH_2 \cdot CH_2CONH_2$$

Mol. wt. 146.1

Submitted by H. B. Vickery and G. W. Pucher, Connecticut Agricultural Experiment Station, New Haven.
Checked by W. L Williams, E. E. Snell, and F. M. Strong, University of Wisconsin, Madison.

I. Principle

The natural glutamine content of the root tissue of the common red beet (*Beta vulgaris*) is increased by treatment of the plants with dilute ammonium sulfate solution. The roots are frozen

[31] P. Fantl and M. N. Rome, *Australian J. Exptl. Biol. Med. Sci.*, 20, 187 (1942).
[32] C. S Hanes, *Proc. Roy. Soc. (London)*, B128, 421 (1939–1940).
[33] C. S. Hanes, *Proc. Roy. Soc. (London)*, B129, 174 (1940).
[34] J. B. Sumner and G. F. Somers, *Arch. Biochem.*, 4, 11 (1944).
[35] P. Bernfeld, C. de Traz, and C. Gautier, *Helv. Chim. Acta*, 27, 843 (1944).
[36] D. E. Green and P. K. Stumpf, *J. Biol. Chem.*, 142, 355 (1942).
[37] R. M. McCready and W. Z. Hassid, *J. Am. Chem. Soc.*, 66, 560 (1944).
[38] C. Weibull and A. Tiselius, *Arkiv Kemi, Mineral. Geol.*, 19A, No. 19, 1 (1945).

and thawed to destroy the permeability of the cells, are ground, and extracted with cold water, and the extract is treated with basic lead acetate to precipitate interfering substances. After filtration, the glutamine is precipitated with mercuric nitrate. The precipitate is decomposed with hydrogen sulfide and filtered, and the solution is neutralized with ammonium hydroxide and concentrated in vacuum at low temperature. The glutamine is crystallized in the presence of 60% alcohol and purified by recrystallization.

II. Starting Material

Enrichment of beet-root tissue by treatment of the plants with ammonium sulfate is not essential but may increase the yield from approximately 2 gm. per kg. of fresh weight to twice this quantity or even more. Ammonium sulfate is applied in 0.2 *M* solution with a watering can or spray to sturdy, well-grown beet plants at the rate of 270 l. per 100 sq. ft. (approximately 1500 lb. of nitrogen per acre), and the roots are harvested 1 week later. Alternatively, the plants may be dug and washed off, and the roots immersed in 0.2 *M* ammonium sulfate solution in pails, approximately 10 l. per kg. of root tissue being used. After 5 to 7 days the leaves and fibrous rootlets are trimmed off, and the roots are washed thoroughly and placed in freezing storage where they may be held almost indefinitely.

In the lack of more suitable material, beets purchased in the market may be used. Old, large beets such as those often discarded from fall gardens as too woody for food purposes have consistently yielded 2 to 3 gm. of glutamine per kg. without any pretreatment with ammonium sulfate. In contrast, small, tender beets purchased in the market have given very poor results when used directly and only fair yields after being soaked in ammonium sulfate solution.

III. Procedure

The following description applies to units of 10 kg. of roots.

The frozen roots are coarsely sliced, preferably with a slicing machine, and are ground through a meat chopper (Hobart mill). The cold pulp is warmed to room temperature, and the juice is

pressed out with a hydraulic press.[1,2] To do this, a convenient quantity of the pulp is wrapped firmly in a square of drilling to form a square package; three or more of these in a pile are pressed at one time between steel plates. The cakes are mixed with 2 l. of water and the pressing is repeated; this washing operation is repeated a second time. The total effluent from the press (13–15 l.) is treated with basic lead acetate reagent [3] in slight excess by test (approximately 800 ml.) and immediately filtered on Büchner funnels fitted with No. 3 Whatman filter paper covered with a thin layer of celite. The precipitate is packed down hard and washed 3 times with water, a total of 6–8 l. being used. When using four 25-cm. funnels, the filtration time is less than 3 hours.

The clear yellow filtrate (20–22 l.) is treated with mercuric nitrate reagent [4] in slight excess as shown by the failure of a filtered test to give more precipitate with a drop of reagent; 1.2–1.4 l. are required, depending on the quantity of glutamine present. The suspension is then neutralized to approximately *p*H 6 (yellow to "Alkacid" test paper) with 10% sodium hydroxide with vigorous stirring. The operations to this point

[1] If it is inconvenient to freeze the roots, cytolysis of the cells may be brought about with ether. The thoroughly ground tissue is treated with sufficient ether to provide a small excess over that necessary to saturate the aqueous phase, and the mixture is stirred and allowed to stand for at least half an hour before expression of the juice.

[2] In the absence of an hydraulic press, a screw press may be substituted or the tissue may even be wrapped in convenient quantities in strong cotton cloth and the juice wrung out by hand. In either method, a liberal volume of wash water should be used if maximal yields are desired.

[3] Basic lead acetate reagent may be prepared by dissolving 180 gm of lead acetate in about 700 ml. of hot water to which 110 gm. of lead oxide (litharge) are then added in powdered form. The mixture is boiled with stirring for half an hour, cooled, filtered, and diluted with water to 1 kg. Alternatively, an approximately 25% solution of commercial basic lead acetate may be used.

[4] Mercuric nitrate reagent may be prepared by slowly adding 220 gm. of high-grade red mercuric oxide to 160 ml. of concentrated nitric acid with stirring; 160 ml. of water are added, and the mixture is boiled under reflux condenser for 3 or 4 hours or until the oxide has completely dissolved. The solution is cooled and treated with *N* sodium hydroxide until a faint permanent opalescence is produced; it is diluted to 1 l. and filtered. It is kept in a dark bottle.

A suitable reagent is obtained even more simply by dissolving mercuric nitrate in approximately 25% concentration in water containing sufficient nitric acid to avoid turbidity, and neutralizing with sodium hydroxide solution to faint opalescence.

require about 6 hours, and it is usually convenient to allow the precipitate to settle overnight. It is desirable that the operations up to this point be carried out at one time, with no more delay than necessary.

The clear supernatant fluid is siphoned off, the precipitate is transferred to two 25-cm. Büchner funnels fitted with No. 3 Whatman paper covered with a thin layer of celite, and is washed 3 times with a total of 4–5 l. of water. If the precipitate is not thoroughly washed, an undue quantity of ammonium hydroxide will be required for the subsequent neutralization.

The precipitate is suspended in the minimum necessary amount of water, all lumps being broken up, and transferred to a 10-l. filter flask. The total volume should be about 3 l. To this suspension, 2.5 ml. of 4 *N* sulfuric acid are added, the flask is placed on a shaking machine, and hydrogen sulfide is passed in under light pressure for about 2 hours with continuous agitation. Air is then passed through the suspension for a short time to remove most of the excess hydrogen sulfide, and the precipitate is filtered and washed with 1–2 l. of water. The clear pale yellow filtrate is transferred to two 5-l. flasks and concentrated in vacuum for about 20 minutes with the bath temperature at 60° to remove the last traces of hydrogen sulfide. The solutions are then recombined and neutralized to approximately *p*H 6 by the careful addition of 15 *N* ammonium hydroxide; from 30 to 50 ml. are required. The solution is then distributed among four 5-l. flasks and concentrated in vacuum [5] with the bath temperature at 60 to 65° to a total volume of about 1 l. The concentrated solutions are combined, treated with a little Norite (or other satisfactory activated charcoal) at 60°, and filtered perfectly clear; concentration is then continued in a 2-l. flask until a sludge of crystals is obtained (volume 200–300 ml.). The total time required for the concentration is about 3 hours. The time may be shortened somewhat by increasing the water-bath temperature

[5] It is important that the time taken for the concentration shall be as short as possible because of the instability (i e , conversion to the ammonium salt of pyrrolidone carboxylic acid) of glutamine at high temperatures. The use of four sets of distillation equipment which may be operated simultaneously is therefore advocated. Attention to this point is vitally necessary if large-scale production is attempted.

especially in the early stages, but this is done at the risk of decomposition of some of the glutamine; losses of the order of 3 to 5% are experienced. When the solution has become fairly concentrated, the temperature should be carefully controlled.

After the vacuum is released the contents of the flask are warmed to 60 to 70°, when most of the crystals dissolve; 2 volumes of warm alcohol are then added, and the flask is chilled overnight. The crystals are filtered, washed with cold 50% ethanol and then with 95% ethanol and ether, and dried over sulfuric acid in a vacuum desiccator.

IV. Purity and Yield of Product

The crude glutamine so obtained is usually about 95% pure as calculated from the nitrogen content (theory 19.18%) or from the amide nitrogen as determined after a 1-hour hydrolysis [6] with 1 *N* acid (theory 9.59%). Asparagine is present only in traces. The chief impurities are ash and a small proportion of an apparently non-nitrogenous organic material of unknown nature. The Molisch reaction is negative, as a rule. Freedom from ammonium salts, in particular the ammonium salt of pyrrolidone carboxylic acid, is tested with Nessler's reagent. There should be no development of color for a minute or so or until traces of ammonia are produced by hydrolysis of glutamine by the alkaline reagent.

The impurity, especially the ash, can be diminished by recrystallization; the crystals are dissolved in about 10 times their weight of water at 60 to 70°, treated with a little decolorizing charcoal, and filtered. Two volumes of warm alcohol are added, and the solution is chilled. The loss on recrystallization is about 10%, the ash is practically eliminated, and the purity is increased to about 97%. Repeated recrystallization from warm water is necessary to raise the purity to 99% or better and can be done only with considerable sacrifice in yield although most of the glutamine can be recovered in impure form from the aqueous mother liquors by treatment with alcohol.

[6] Analytical results for amide nitrogen of sufficient accuracy to serve as convincing evidence of purity can be obtained only by hydrolysis in the presence of acid.

The quantity of glutamine obtained depends upon the richness of the original tissue. From 10 kg. of beet roots that had been treated with ammonium sulfate in the field, approximately 35 gm. of crude material have been obtained. The solution from the decomposition of the mercury precipitate contained from 90 to 92% of the glutamine in the extract from the tissue, and about 97% of this was isolated as crude crystals of nitrogen content 18.27% (95.3% pure) and amide nitrogen content of 9.07% (94.5% pure); the ash content was 0.93%, and approximately 0.3% of asparagine was detected by a specific analytical method.

It should be noted that mercuric nitrate is by no means a specific precipitant for glutamine. Asparagine is also precipitated quantitatively and is present in small amounts in the precipitate obtained from the extract of beet roots. Small amounts of other amino acids have been identified in the precipitate from other tissues, notably arginine, lysine, tyrosine, and cystine; adenine may also be present.

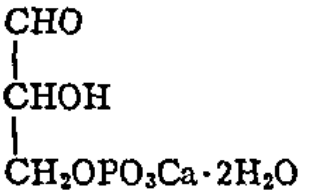
V. Methods of Preparation

The present method is a modification as regards apparatus and technique of the method originally described by Schulze and Bosshard [7] and a slight simplification of the procedure of Vickery, Pucher, and Clark.[8] Glutamine can be obtained from a number of plant tissues other than beet roots, but this material is the best known and the most readily available as well as one of the richest if properly prepared. Furthermore, most tissues also contain considerable asparagine which can be separated from glutamine only by a troublesome fractional crystallization procedure. The quantity of asparagine in beet roots is, ordinarily, so small as not to interfere. Synthetic methods to prepare L-glutamine from L-glutamic acid have been described,[9,10] but neither of these methods is as convenient for large-scale work as isolation from natural sources.

[7] E. Schulze and E. Bosshard, *Landw. Versuch. Sta*, 29, 295 (1883).
[8] H. B. Vickery, G. W. Pucher, and H. E Clark, *J. Biol Chem*, 109, 39 (1936)
[9] M Bergmann, L. Zervas, and L Salzmann, *Ber.*, 66, 1288 (1933).
[10] J. S Fruton, *J. Biol. Chem.*, 165, 333 (1946).

DL-GLYCERALDEHYDE 3-PHOSPHORIC ACID

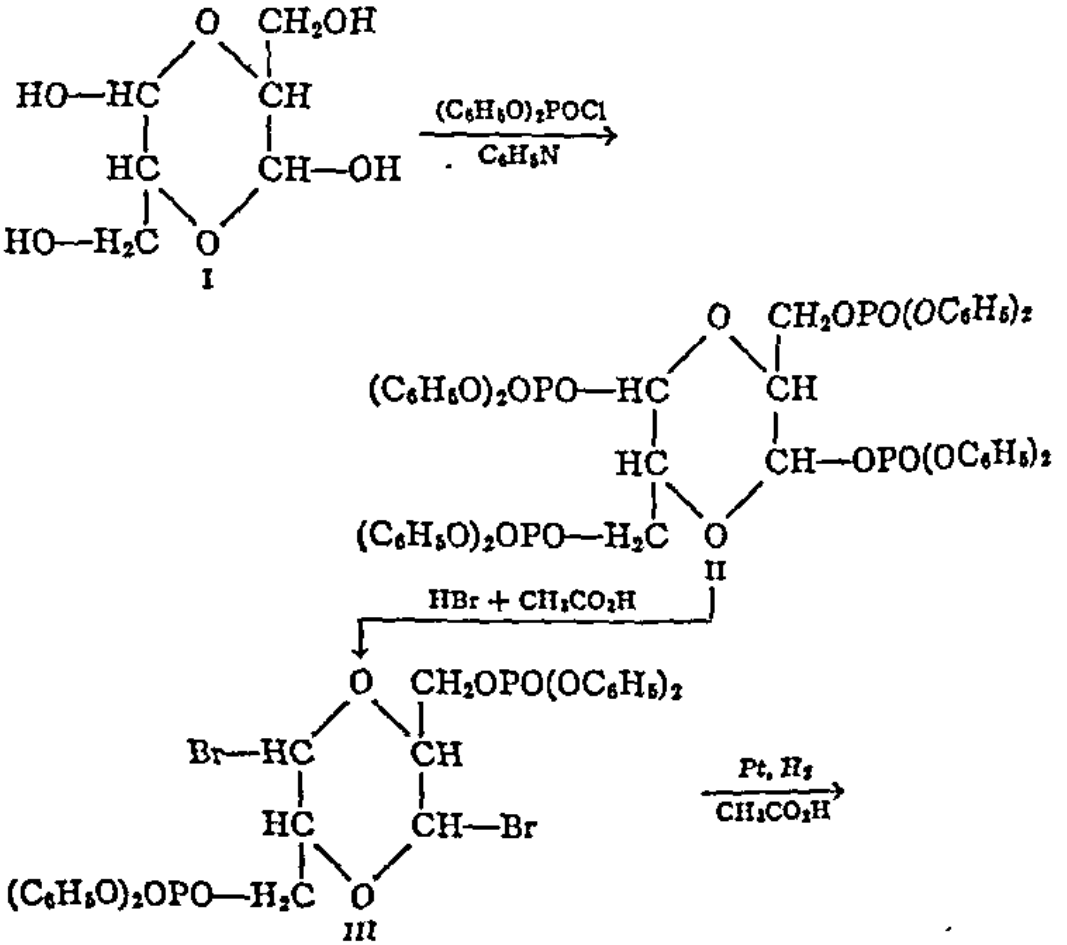
(Calcium salt, dihydrate)

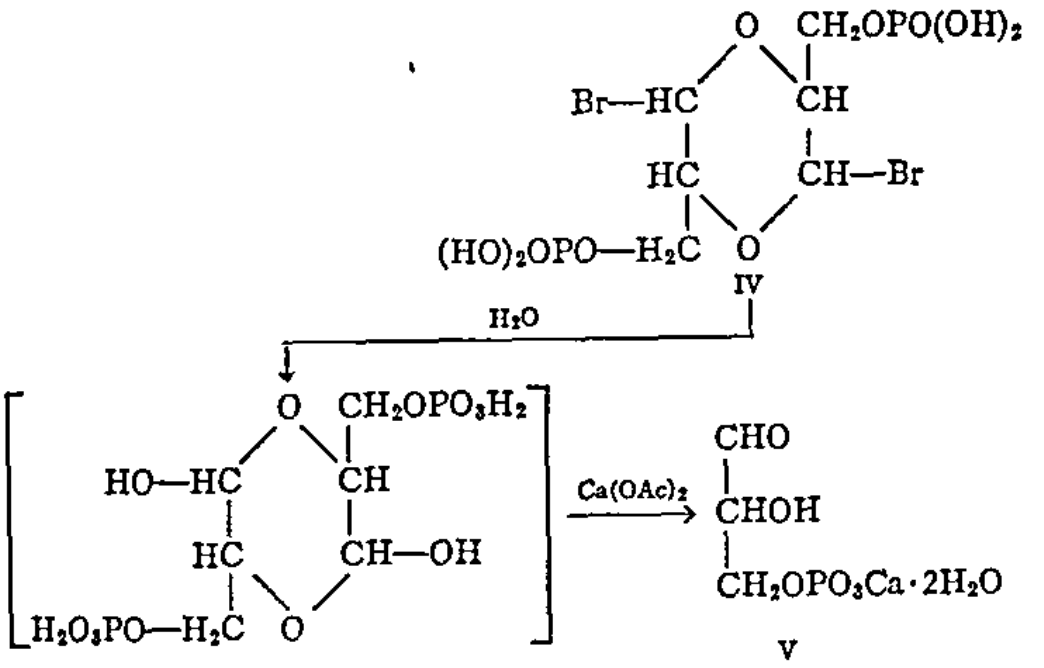


Mol. wt. 244.2 ($C_3H_5O_6PCa \cdot 2H_2O$)

Submitted by ERICH BAER, Banting Institute, Toronto.
Checked by HENRY LARDY, University of Wisconsin, Madison.

I. Principle





II. Starting Material

DL-Glyceraldehyde is not readily available commercially. The crude product obtained by the procedure of Witzemann et al.[1] is refluxed with 8 times its weight of dry acetone for 30 minutes, filtered, ground finely, and passed through a 200-mesh sieve (m.p. 141–142°).

Diphenylchlorophosphate (diphenylphosphoryl chloride) is prepared by a slight modification of the procedure of Brigl and Müller.[2] One hundred and sixty-eight grams (1.1 moles) of phosphorus oxychloride and 188 gm. (2 moles) of best-grade phenol are placed in a 1-l. round-bottomed flask fitted with a reflux condenser and thermometer. The condenser is connected to a sodium hydroxide trap for the absorption of hydrogen chloride and phosphorus oxychloride. The mixture is heated to gentle boiling with an electric heater until the temperature reaches 180° (about 2 hours). The reaction mixture is transferred to a Claisen flask and fractionally distilled at a low pres-

[1] E. J. Witzemann, W. L. Evans, H. Hass, and E F Schroeder, *Org. Syntheses* Coll. Vol. 2, 305 (1943). An improved method of preparation for one of the intermediates—acrolein acetal—is described by H. O. L. Fischer and E. Baer, *Helv. Chim. Acta*, 18, 514 (1935).

[2] P. Brigl and H. Müller, *Ber.*, 72, 2121 (1939)

sure. The fraction distilling at 140–155°/1.3 mm. is collected and redistilled. The material distilling at 147–148°/1.3 mm. is essentially pure diphenylchlorophosphate. The yield is 200–212 gm. (74–79%).

III. Procedure

A. DL-Glyceraldehyde 1,3-bisdiphenylphosphate (Dimeric) (II). A 200-ml. two-necked flask, equipped with a mercury-sealed, motor-driven stirrer, is placed in a water bath maintained at a temperature of 10–15°. The stirrer should be of such a design that the entire reaction mixture is thoroughly stirred throughout the phosphorylation. The stirrer is started, and through the second opening are added in the following sequence 22.5 gm. of ice-cold dry pyridine,[3] 66.5 gm. of ice-cold diphenylchlorophosphate, and 10.8 gm. of dry, finely powdered glyceraldehyde. The flask is closed and vigorous stirring is continued for 6 hours, care being taken that the temperature of the mixture, which soon becomes a stiff paste, does not rise above that of the water bath.[4] At the end of 6 hours the stirrer is stopped and the pastry mixture is allowed to stand in the water bath at room temperature for an additional 18 hours. The almost solid reaction mixture is cooled in an ice bath and stirred while 150 ml. of ice-cold 95% ethanol are added slowly. All lumps are broken up, and the thin sludge is filtered with suction. The residue is washed on the filter with small volumes of ice-cold ethanol until colorless. The crude triosephosphate ester is dissolved in 75 ml. of luke-warm dioxane, and a small quantity of unreacted glyceraldehyde is removed by filtration. Petroleum ether (b.p. 80–100°) is added gradually with scratching to initiate crystallization of the product. Care must be exercised at this stage to avoid formation of an oil. A total of 450 ml. of petroleum ether is added, and the crystalline precipitate is filtered giving 21 gm. (39% yield) of pure glyceraldehyde 1,3-bisdiphenylphosphate melting at 110–111°. This substance is readily soluble in cold

[3] The best grade of reagent pyridine is dried over potassium hydroxide sticks and distilled.

[4] If the temperature rises, strong discoloration results and the yield is decreased.

dioxane and chloroform and in warm ethyl acetate, benzene, and carbon tetrachloride.

B. DL-Glyceraldehyde 1-bromide 3-diphenylphosphate (Dimeric) (III). Twenty-one grams of finely powdered DL-glyceraldehyde 1,3-bisdiphenylphosphate are placed in an Erlenmeyer flask with a ground-glass stopper, and 75 ml. of an ice-cold solution of anhydrous hydrobromic acid (30–32%) in glacial acetic acid (Eastman Kodak Company) are added. The stoppered flask is gently shaken until the phosphate is dissolved (5 minutes) and then is set aside at room temperature. Well-formed crystals of the bromo compound (III) usually appear after 30 minutes' standing. At the end of 24 hours the crystalline paste is poured into 500 ml. of a vigorously stirred mixture of ice and water (1:2), and the stirring is continued for 10 minutes. The bromo compound (III) is filtered with suction, washed on the filter with ice-cold water, and dried in vacuum over calcium chloride. The crude product, weighing usually about 13.7 gm. (94% yield), is recrystallized from 400 ml. of boiling ethyl acetate [5] yielding 11.4 gm. (78%) of pure DL-glyceraldehyde 1-bromide 3-diphenylphosphate (m.p. 161–162°). The substance is readily soluble in warm benzene, toluene, carbon tetrachloride, ethyl acetate, acetone, and dioxane but is only sparingly soluble in warm ether or petroleum ether.

C. DL-Glyceraldehyde 1-bromide 3-phosphoric acid (Dimeric) (IV). The cleavage of the phenyl ester by catalytic reduction can be carried out in almost any apparatus designed for low-pressure hydrogenation. A simple arrangement is described in Gattermann.[6] Eleven and four-tenths grams of finely powdered DL-glyceraldehyde 1-bromide 3-diphenylphosphate are suspended in 250 ml. of pure, dry acetic acid [7] and reduced at

[5] Ordinary ethyl acetate must be purified by washing successively with an equal volume of 5% sodium carbonate solution and saturated calcium chloride solution, drying over potassium carbonate or phosphorus pentoxide, and distilling.

[6] *Laboratory Methods of Organic Chemistry*, English edition, p. 376, Macmillan and Company, 1938.

[7] Glacial acetic acid is boiled under reflux for several hours with solid potassium permanganate or chromic anhydride, distilled, and kept over Drierite. Traces of the drying agent are removed by centrifuging the acid just before use. The success of the reduction depends to a large extent on the purity and dryness of the acetic acid.

room temperature [8] with 3.0 gm. of freshly prepared platinum oxide [9] under dry hydrogen [10] at a pressure of 60 cm. of water. Disappearance of the insoluble phenyl ester indicates the approaching end of the reduction. The shaking is continued until 10% more than the theoretical amount of hydrogen (16 moles) is taken up (5–6 hours). The hydrogen is expelled with nitrogen, the clear solution is filtered, and the catalyst is washed several times with a few milliliters of dry acetic acid.[11] The combined filtrates are concentrated in vacuum (10 mm.) at a bath temperature not exceeding 25°.[12] The solid residue [13] is triturated with 20 ml. of dry acetone, filtered with suction, and washed on the filter with three 10-ml. portions of acetic acid. The residue is dried in vacuum over phosphorus pentoxide and solid sodium hydroxide, giving 5.45 gm. (79% yield) of glyceraldehyde 1-bromide 3-phosphoric acid which is 90–92% pure based on the determination of total and alkali-labile phosphate. This crude product is purified further with difficulty but readily yields an analytically pure dioxane addition compound.

D. Dioxane addition compound of DL-glyceraldehyde 1-bromide 3-phosphoric acid (Dimeric). To a clear solution of 5.45 gm. of the crystalline glyceraldehyde 1-bromide 3-phosphoric acid in 30 ml. of tributyl phosphate (Commercial Solvents Company) are added 60 ml. of purified dioxane, and the mixture is

[8] The temperature of the reduction mixture is maintained at 20–25° by occasionally blowing ether on the reaction vessel.

[9] R. Adams, V. Voorhees, and R. L. Shriner, *Org. Syntheses* Coll. Vol. 1, 463, 2nd edition, 1941. The time of the reduction is shortened by using larger amounts of catalyst. However, such an increase seems to favor formation of non-cleavable cyclohexyl esters which are difficult to remove. Their presence in the final product is indicated by high carbon values.

[10] A calcium chloride tube or acetone-Dry Ice trap may be used to dry the hydrogen.

[11] The catalyst may ignite the filter paper if it is sucked dry.

[12] The use of a round-bottomed flask with a short standard taper neck facilitates removal of the solid residue. The capillary inlet tube and the side arm of the receiver should be fitted with calcium chloride tubes to protect the solution from moisture.

[13] If the residue fails to solidify it is dissolved in 10–15 volumes of dioxane and the solution is filtered and stored in the icebox for several days. The dioxane addition compound is obtained in a smaller yield but in as high a state of purity as that prepared from crystallized glyceraldehyde 1-bromide 3-phosphoric acid.

allowed to stand undisturbed [14] for 24 hours at room temperature, during which time the dioxane compound crystallizes in long, narrow prisms. The mother liquor is carefully decanted, and the precipitate is washed on the filter with cold dioxane and dried in high vacuum over solid sodium hydroxide, giving 6.54 gm. (87% yield) of the dioxane addition compound of DL-glyceraldehyde 1-bromide 3-phosphoric acid. This compound contains 2 moles of dioxane per mole of bromide. It is free of inorganic phosphate and hydrolyzes readily to DL-glyceraldehyde 3-phosphoric acid.

E. Calcium salt of DL-glyceraldehyde 3-phosphoric acid (V). An ice-cold solution of 6.54 gm. of the dioxane compound in 21 ml. of water is added to a cold solution of 7.2 gm. of calcium acetate (anhydrous) in 36 ml. of water, and the mixture is filtered immediately. The filtrate is cooled in an ice bath and gently stirred during the dropwise addition of 42 ml. of ethanol over a period of 30 minutes. The ethanol must be added slowly, especially in the early stages, to ensure the separation of a nicely crystalline calcium salt. The calcium salt is filtered, washed 3 times with 20-ml. portions of ice-cold 40% ethanol, and sucked as dry as possible. The air-dried material is ground and dried at room temperature in vacuum (8–10 mm.) over calcium chloride for 2 hours, giving glyceraldehyde 3-phosphoric acid calcium salt dihydrate. The yield is 3.14 gm. (63%) of material containing only traces of inorganic phosphate.

IV. Properties and Purity of Product

The purity of the triose phosphate can be determined by analysis of the total and alkali-labile phosphorus,[15] by the rate of acid hydrolysis,[15] by the Willstätter-Schudel titration,[15] and by formation of methylglyoxal.[15] The calcium salt is unstable, particularly in alkaline solution, liberating inorganic phosphate.

The dioxane addition compound of DL-glyceraldehyde 1-bromide 3-phosphoric acid is relatively stable and can be stored

[14] Slow crystallization gives a better product.

[15] E. Baer and H. O. L. Fischer, *J. Biol. Chem.*, 150, 223 (1943).

for some time if kept dry and cold. After 2 months in the icebox less than 2% of inorganic phosphate is formed. If the preparation is carried through on a scale larger than the immediate needs, the product should be held as the dioxane addition compound.

The checkers point out that dioxane and bromide ion do not interfere with the isomerase-aldolase or the triose phosphate dehydrogenase systems of rat-muscle extract, which are commonly used in studying glyceraldehyde 3-phosphoric acid metabolism, whereas calcium ion (and oxalate, which may be used to remove calcium) are frequently toxic to these systems. They suggest in view of these facts and of the stability of the dioxane compound of glyceraldehyde 1-bromide 3-phosphoric acid that this substance is the compound of choice for biochemical work. An aqueous solution of the sodium (or potassium) salt of glyceraldehyde 3-phosphoric acid (containing dioxane and bromide ion) is readily prepared by dissolving the dioxane compound in cold water and adding alkali to *p*H 7. Care should be exercised to avoid adding excess alkali since glyceraldehyde 3-phosphoric acid is particularly labile in alkaline solution.

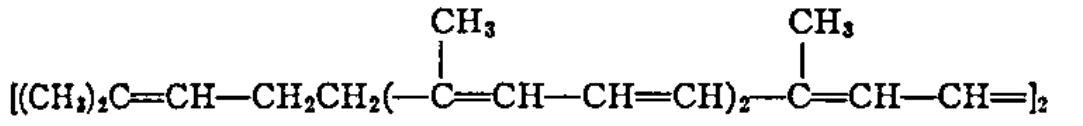
V. Methods of Preparation

DL-Glyceraldehyde 3-phosphoric acid has been prepared by the hydrolysis of dimeric glyceraldehyde 1,3-diphosphoric acid [16] and of dimeric glyceraldehyde 1-bromide 3-phosphoric acid.[15] It has also been prepared by an extended series of reactions terminating in DL-glyceraldehyde 1-benzyl ether 3-phosphoric acid (dimeric) which on reductive cleavage of the benzyl ether group gives DL-glyceraldehyde 3-phosphoric acid.[17]

D-Glyceraldehyde 3-phosphoric acid ($[\alpha]_{5460} = +24°$; $[\alpha]_D = +19°$) has been prepared enzymatically by intercepting the triose phosphate with hydrazine.[18]

[16] E. Baer and H. O. L. Fischer, *J. Biol. Chem.*, 150, 213 (1943).
[17] H. O. L. Fischer and E. Baer, *Ber.*, 65, 337, 1040 (1932).
[18] O. Meyerhof and R. Junowicz-Kocholaty, *J. Biol. Chem.*, 149, 71 (1943).

LYCOPENE



Mol. wt. 536.85 ($C_{40}H_{56}$)

Submitted by A. SANDOVAL and L. ZECHMEISTER, California Institute of Technology, Pasadena.
Checked by W. J. FRAJOLA, C. S. VESTLING, and H. E. CARTER, University of Illinois, Urbana.

I. Principle

Tomato paste is dehydrated with methanol, and lycopene is extracted from the residue with methanol-carbon tetrachloride. The crude product is crystallized twice from benzene by the addition of methanol, giving lycopene of 98–99% purity. Further purification is achieved by a chromatographic procedure, using calcium hydroxide as the adsorbent.

II. Starting Material

Canned tomato paste may be obtained from a local grocer. It should be noted that paste (not purée or tomato conserve) is used.

III. Procedure

A. Extraction of lycopene. Canned tomato paste is dehydrated by adding 1.3 l. of methanol to 1 kg. of the paste [1] contained in a 5-l. wide-mouthed bottle. The mixture is shaken vigorously and without delay; otherwise hard lumps will form. A small sample of the suspension is tested by hand; if it has a gluish consistency, more methanol is added to the main portion

[1] The checkers used 6 cans of "Firenze" paste, 6 ounces net, or a total of 1.01 kg. This paste is produced by the Turlock Cooperative Growers of San Francisco.

in order to avoid the possible clogging of filters. The mixture is allowed to stand for 1–2 hours and is then shaken vigorously. The thick suspension is separated in a basket centrifuge (diameter, 20 cm.), using a piece of cloth as filter. If such a centrifuge is not available, a Büchner funnel (diameter, 20 cm.) with filter paper can be used.[2] The yellow filtrate is discarded.

The dark red cake is returned to the bottle and shaken with a mixture of 650 ml. of methanol and 650 ml. of carbon tetrachloride. The cap of the bottle must fit well and should be opened for a moment after the mixing, in order to release any pressure. A short shaking and opening of the bottle are repeated until no more excess pressure is noticed. The suspension is shaken mechanically for 20 minutes and separated in the basket centrifuge (or by filtration on a large Büchner funnel [2]). The filtrate consists of a lower, very dark red, carbon tetrachloride phase and an orange aqueous-methanolic layer. The only moderately colored tomato residue is crushed by hand to form a nearly uniform powder. It is then re-extracted with 650 ml. of each solvent as described, and the suspension is filtered. The extraction is now practically complete. A sample of the residue, when dried on filter paper, should appear colorless or yellowish but not red. If necessary, a third extraction is made.

The filtrates are combined, and the methanol layer (or as much of it as is convenient) is transferred to a 4-l. separatory funnel, and 1 volume of water is added. A white emulsion appears in the upper phase. If the emulsion is reddish, it is stirred with a glass rod until the droplets of carbon tetrachloride join the lower layer. The upper phase is then siphoned off as far as possible and the process is repeated until all the extract (methanol and carbon tetrachloride phases) has been added to the separatory funnel. The remaining deep red solution is freed of methanol and other impurities by introducing a glass tube (0.5-cm. diameter) vertically to the bottom of the separatory funnel and letting a slow stream of water flow through it for 10 minutes. (The funnel can be hung over a sink, and the water allowed to overflow along

[2] No filter aid should be employed.

its outside walls.) The carbon tetrachloride solution is drained into a 2-l. Erlenmeyer flask and dried over anhydrous sodium sulfate. The extract is then poured through a folded filter into a 2-l. round-bottomed flask equipped with a standard taper. The solvent is evaporated on the water pump [3] to about 100 ml. in a water bath at 60°. The solution is pipetted into a similar flask of 200-ml. capacity using a few milliliters of carbon tetrachloride to rinse the larger flask. The solvent is then removed completely in vacuum.[3]

B. Purification of crude product. The dark oily residue from A is diluted with a few milliliters of benzene and evaporated again in order to eliminate the carbon tetrachloride completely. The partly crystalline, dark residue is transferred quantitatively with 25 ml. of benzene into a 125-ml. Erlenmeyer flask. By immersing the flask in a hot water bath, a clear solution is obtained.[4] Boiling methanol is added in portions, with a dropper, to the benzene solution, with stirring after each addition, until 15 ml. of methanol is introduced. Crystals of crude lycopene begin to appear immediately. The crystallization is completed by keeping the liquid first at room temperature and then in ice water. After standing for 1 to 2 hours the crystals are collected in a small fritted-glass funnel of *medium* porosity. In order to eliminate some impurities, the suction is stopped, 5–10 ml. of boiling methanol are poured on the crystals, and, after a very short stirring with a glass rod, the suction is renewed before the methanol cools. This treatment should be repeated until a total of about 50 ml. of methanol has been used. The combined mother liquors and washings are discarded in spite of their dark red color, since a large amount of carotenes and impurities would prevent the crystallization of pure lycopene from this source. The amount of lycopene in these mother liquors is of the order

[3] The checkers found that the distillation proceeded more smoothly if carbon dioxide was admitted through a capillary tube.

[4] All steps in which lycopene solutions are involved should be carried through rapidly. If lycopene solutions are kept overnight they should be stored in a cold room.

of 10% of the total present in the extract, and to attempt recovery of this material.

The lycopene crystals are transferred to a trifuge tube, the last portion being dissolved with small quantities of boiling benzene. Ben the centrifuge tube to make a total volume of 25 the crystals is obtained by dipping the tube int stirring the contents. If a clear solution is no milliliters of benzene are added. When a clea tained,[4] boiling methanol is introduced with a d portions, and the solution is stirred with a glass r begin to appear. The centrifuge tube is then temperature for a short time and then in an i methanol is added in small portions with stirri solution. The total volume of methanol present ceed 20 ml.

The mixture is allowed to stand for 2 hours in t the crystals are separated by a brief but stron The mother liquor is decanted and discarded. T treated in the centrifuge tube with 25 ml. of boi The mixture is stirred, and the methanol is remo fuging before it cools. The methanol is decanted, a is repeated at least twice more. If the crystalliza fication were satisfactory, a homogeneous structur long, red lycopene prisms is observed under the mi colorless substance should be present. The centrif its contents are dried in vacuum (0.5–1.0 mm.) in the apparatus at room temperature for a few hours and The yield, which is dependent on the quality of the t was found in four experiments with the same batch o 246 mg., 263 mg., 212 mg., and 268 mg. of materia 169° (cor. Berl-block in a sealed capillary tube). A analyses, such a lycopene sample is 98–99% pure.

As shown by the older literature, it is possible to ob lytically pure lycopene by means of further recrystal However, the following chromatographic procedure satisfactory.

C. Purification of lycopene by chromatography. A calcium hydroxide [5] column (30 by 8 cm.; tube 33 by 8 cm.[6]) is prepared. The sample is then dissolved in 50 ml. of benzene by a short and gentle heating (not above 40–45°). This solution is diluted with 1 volume of petroleum ether (b.p. 60–70°) and poured onto the column without delay. The chromatogram is developed with about 1.5 l. of 4:1 petroleum ether-acetone. However, the volume and the ratio (4:1) given will vary with the quality of the calcium hydroxide used. The figures on the left in the following sequence denote the width of the zones in millimeters and may vary in different experiments.

25 almost colorless
35 pale pink
85 deep orange-red; lycopene
3 orange: neolycopenes
7 yellow: neolycopenes
Filtrate: yellow

During the first stage of the development, a dark red zone may appear near the top of the lycopene zone. This is caused by a temporary crystallization of the pigment and disappears again in the course of the development.

The column is extruded; [7] the lycopene zone is cut out, chopped rapidly with a knife, and transferred to a 2-l. wide-mouthed Erlenmeyer flask where it is covered immediately with acetone in order to elute the pigment. After a few minutes of gentle shaking, the homogeneous suspension is poured under suction on a fritted-glass filter (best diameter, 12.5 cm.). Fresh acetone is introduced until both the flow and the adsorbent become almost colorless; a total volume of 2 l. of acetone is usually adequate.

The acetone solution is added in the following manner to 200

[5] Shell Brand lime, chemical hydrate, 98%, or calcium hydroxide prepared according to Zechmeister and Cholnoky, *Principles and Practice of Chromatography*, 2nd edition, p 49, translated by Bacharach and Robinson. The tube is prepared as described on p. 69 of the above reference.

[6] Suitable chromatographic tubes are available from the Scientific Glass Apparatus Company, Bloomfield, New Jersey.

[7] Difficulty may be encountered if the tube is sucked too dry.

ml. of benzene, contained in a 2-l. separatory funnel. First, sufficient of the acetone solution is introduced to bring the liquid to half the volume of the funnel. At this point 1 volume of water is added which transfers the pigment into the upper (benzene) phase. After a few minutes the colorless lower layer (aqueous-acetone) is drained off and discarded, and a new portion of the lycopene solution is introduced as above. Thus, finally, the total pigment will be present in about 350 ml. of benzene-petroleum ether (the latter solvent originating from the eluate).

The solution is washed acetone-free by shaking 10 times with water [8] and is transferred to a 750-ml. Erlenmeyer flask and dried over sodium sulfate. It is then poured through a gravity paper filter into a 500-ml. round-bottomed flask (with standard taper) and the solvent is completely removed in vacuum. The crystals are quantitatively transferred with 15 ml. of benzene into a weighed 50-ml. centrifuge tube, and the lycopene is crystallized out as described above, by using 15 ml. of methanol. The treatment of the crystals can be carried out with ice-cold methanol in this case. About 70% of the crude lycopene is recovered as pure lycopene melting at 173° (cor. Berl-block).

IV. Properties and Purity of Product

Lycopene melts at 173° (cor. Berl-block). It has absorption maxima in petroleum ether at 446, 474, and 505 mμ [9] and an E_m (472–473 mμ) = 18.6×10^4 in hexane.[10] Lycopene in solution undergoes isomerization even at 20°.[11] Crystalline lycopene does not isomerize but has a tendency to autoxidize, especially in light. It should be kept in the dark in evacuated glass tubes. If this is not feasible it may be stored under dry, acid-free nitrogen or carbon dioxide. The tube should alternately be evacuated and flushed out with the gas before sealing.

[8] A continuous apparatus such as described by A. L. Le Rosen, *Ind. Eng. Chem.*, 14, 165 (1942), may be used.

[9] A. L. Le Rosen and L. Zechmeister, *J. Am. Chem. Soc.*, 64, 1075 (1942). See also F. P. Zscheile and J. W. Porter, *Ind. Eng. Chem., Anal. Ed.*, 19, 47 (1947).

[10] L. Zechmeister et al., *J. Am. Chem. Soc.*, 65, 1940 (1943).

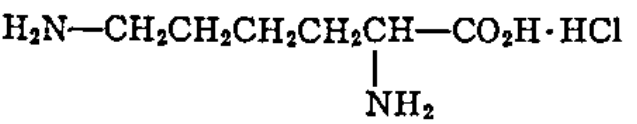
[11] L. Zechmeister and P. Tuzson, *Biochem. J.*, 32, 1305 (1938).

V. Methods of Preparation

Lycopene has been prepared from a variety of fruits and berries.[12] The first modern preparation is that of Willstätter and Escher,[13] who isolated lycopene from tomatoes. The general procedure involves drying the material with methanol and extracting the lycopene with carbon disulfide [12] or with methanol-petroleum ether.[9]

Some essential features of the present procedure have not been described previously; other steps are similar to those by Le Rosen and Zechmeister in the preparation of prolycopene from tangerine tomatoes. Porter and Zscheile [14] have described the chromatographic separation of the carotenes from various species and strains of tomatoes on a magnesium oxide-Super Cel column.

L-LYSINE MONOHYDROCHLORIDE



Mol. wt. 182 6 ($C_6H_{15}N_2O_2Cl$)

Submitted by ELDON E. RICE, Swift and Company, Chicago, Illinois
Checked by W. D. CELMER and H. E. CARTER, University of Illinois, Urbana.

I. Principle

An appropriate protein is hydrolyzed with sulfuric acid, and lysine is precipitated from the hydrolysate as the insoluble monopicrate. The picrate is converted into the dihydrochloride with hydrochloric acid, and L-lysine monohydrochloride is obtained by treating the dihydrochloride with pyridine.

[12] L. Zechmeister and L. v. Cholnoky, *Ber.*, 63, 422 (1930); J. Zimmerman, *Rec. trav. chim.*, 51, 1001 (1933); P. Karrer, F. Rübel, and F. M. Strong, *Helv. Chim. Acta*, 19, 28 (1936); R. Kuhn, H. Bielig, and O. Dann, *Ber.*, 73, 1080 (1940), etc.
[13] R. Willstätter and H. H. Escher, *Z. physiol. Chem.*, 64, 47 (1910).
[14] J. W. Porter and F. P. Zscheile, *Arch. Biochem.*, 10, 537 (1946).

II. Starting Material

Blood corpuscle paste, prepared by centrifuging whole blood, is the most readily available starting material. This may be prepared from fresh blood or purchased from a meat-packing plant. In the latter case it should be shipped under toluene to retard decomposition. The yield of lysine increases with the erythrocyte content of such preparations.

Dried blood fibrin is equally satisfactory. Although it is a more expensive source of lysine it has the advantage of being more readily shipped and stored.

III. Procedure

A. Hydrolysis. To 4 l. of blood corpuscle paste (or 2.5 kg. of dried blood fibrin) in a 12-l. round-bottomed flask are added slowly and with stirring 4 l. of a mixture of equal volumes of concentrated sulfuric acid and water. The mixture is refluxed for 18 hours and is cooled and diluted with 8 l. of water in a 15-gal. crock. Most of the acid is neutralized with 2.5 kg. of commercial lime suspended in 4 l. of water. The precipitate is removed on a 25- to 30-cm. Büchner funnel and is stirred thoroughly with 2–4 l. of water in a large beaker. The mixture is returned to the Büchner funnel and filtered. The washing process is repeated once more. The combined filtrate and washings are concentrated on the water pump to 8–10 l. and neutralized to *p*H 7 with lime or lime water. The precipitate ($CaSO_4 \cdot 2H_2O$ and less-soluble amino acids) is filtered, and the filtrate is concentrated to about 4 l. on the water pump. (Separation of insoluble amino acids makes further concentration difficult.) The mixture is cooled for at least 12 hours at 0–5°. The precipitate is filtered and washed several times with 50- to 100-ml. portions of cold water. The filtrate and washings are concentrated on the water pump to 1.6–2 l., and the mixture is cooled at 0–5° for at least 12 hours. The precipitate is filtered [1] and

[1] If the concentration is pushed too far the chilled mixture will be a heavy viscous mass which cannot be filtered. If this happens add 200–400 ml. of water, warm to 80°, then cool for 12 hours and filter.

washed several times with 25- to 50-ml. portions of cold water.

B. Precipitation of lysine monopicrate. The hydrolysate is warmed to 80°, and 150 gm. of picric acid [2] are added, with stirring. The solution is cooled to room temperature and then cooled to 0–5° for 12 hours. If the picrate forms slowly the flask is scratched with a glass rod or seeded with crystals of lysine monopicrate and allowed to stand for another day. The crude picrate is filtered on a 6- to 8-in. Büchner funnel and is washed 3 times with 50- to 100-ml. portions of cold water. The crude product is transferred to a 5-l. flask, and 70 gm. of decolorizing charcoal and 1.5 l. of water are added. The mixture is boiled for 5 minutes, and the solution is filtered through a hot Büchner funnel.[3] The charcoal is washed twice with 50-ml. portions of boiling water. The filtrate is cooled to 0–5°, and the lysine monopicrate is filtered.[4] The yield of air-dried product is 90–130 gm. The picrate should melt at 261–265°. If the melting point is lower or if the filtrate is not a clear yellow, the purification with charcoal is repeated.

C. Conversion of lysine picrate to lysine dihydrochloride. The lysine monopicrate is heated on a steam cone for 30 minutes with 300 ml. of water and 300 ml. of concentrated hydrochloric acid. The mixture is stirred frequently. The mixture is cooled, transferred to a separatory funnel, and extracted with ether to remove the picric acid. (If desired much of the picric acid may be removed before the extraction by filtering the mixture through a pad of asbestos fibers.) The aqueous solution is decolorized by boiling for several minutes with 25 gm. of charcoal. The solution is filtered into a 3-l. flask. If the filtrate is not colorless, the charcoal treatment is repeated.

The filtrate is concentrated on the water pump to a thick syrup. The syrup is dissolved with warming in the minimum

[2] The use of excess picric acid causes the yield to be reduced owing to formation of the more soluble dipicrate.

[3] If the funnel is not very hot, lysine monopicrate will crystallize in the funnel and stop the filtration. The funnel should be heated with steam for several minutes before use.

[4] The recrystallization is essential if a histidine-free product is desired.

amount of ethanol. (Usually 300–500 ml. of ethanol are required.) The solution is chilled in an ice-salt bath, and ether is slowly added, with vigorous stirring, until a permanent turbidity is produced. If crystals do not form the flask is scratched with a glass rod until crystallization occurs. The solution is stirred vigorously, and ether is run in slowly until a total of 5 volumes of ether is added. The mixture is placed in the refrigerator overnight, and the lysine dihydrochloride is filtered, washed with ether, and air-dried. The yield is 50–70 gm.

D. Conversine of lysine dihydrochloride to the monohydrochloride. The dihydrochloride is dissolved in the minimum amount of boiling ethanol, and a hot solution of pyridine (0.45 gm. per gm. of dihydrochloride) in ethanol (60 gm. of pyridine per 100 ml. of ethanol) is added with stirring. The flask is placed in the refrigerator overnight, and the crude monohydrochloride is filtered and washed with ethanol and then with ether. The crude material is dissolved in an equal weight of water, and 3 volumes of ethanol are added slowly. The ethanol must be added carefully in order to promote crystallization, but the addition must be completed within 10 minutes; otherwise some lysine monohydrochloride dihydrate precipitates. The mixture is cooled for 12 hours, and the purified lysine monohydrochloride is filtered, washed with absolute ethanol and ether, and dried in vacuum over phosphorus pentoxide.[5] The yield is 40–55 gm., varying with the concentration of erythrocytes in the starting material as well as with the conditions of isolation.

IV. Properties and Purity of Product

L-Lysine monohydrochloride melts at 263–264° and has a rotation of +14.6 in 0.6 *N* hydrochloric acid, calculated on the basis of free lysine. The material obtained in this preparation gives a negative test for arginine and histidine and has the correct optical rotation and nitrogen content.

[5] In one laboratory the final air-dried product was found to analyze for the monohydrate The checkers obtained anhydrous lysine monochloride from aqueous ethanolic solutions but were able to prepare the dihydrate (well-formed, large crystals, m.p. 256–258°) from aqueous solutions. The dihydrate readily loses water over phosphorus pentoxide in vacuum, giving anhydrous material.

V. Methods of Preparation

The oldest method for isolating lysine from protein hydrolysates is that of Kossel and Kutscher,[6] involving the use of silver salts and phosphotungstic acid in an effort to effect a quantitative isolation of lysine. Many modifications of this method have been employed, but none are practical for the preparative isolation of lysine. The electrical transport method [7] has been used successfully but is longer and more complicated than the present procedure. The use of activated fuller's earth and of synthetic ion exchange resins in the isolation of crude lysine has been described.[8] Lysine is isolated from the crude product as the picrate.

The present procedure is essentially that of Rice.[9]

LYSOZYME

(Crystalline: from Egg White)

Mol. wt. *ca.* 17,500

Submitted by H L. Fevold and G. Alderton, Western Regional Research Laboratory, Albany, California.
Checked by L. S. Ciereszko, University of Illinois, Urbana.

I. Principle

A. Adsorption procedure.[1] Lysozyme (Globulin G_1) [2] is adsorbed on bentonite together with other egg white globulins. The contaminating proteins are removed from the bentonite by washing successively with alkaline phosphate and with 5% aqueous pyridine. The lysozyme is then eluted from the bentonite with

[6] A. Kossel and F. Kutscher, *Z. physiol. Chem*, **31**, 165 (1900–01).
[7] G J Cox, H King, and C. P. Berg, *J. Biol. Chem*, **81**, 755 (1929).
[8] R. J. Block, *Proc Soc. Exptl Biol. Med*, **51**, 252 (1942)
[9] E. E Rice, *J. Biol Chem*, **131**, 1 (1939)

LYSOZYME

[1] G Alderton, W. R Ward, and H L. Fevold, *J. Biol Chem*, **157**, 43 (1945)
[2] L G. Longsworth, R K. Cannan, and D A. MacInnes, *J. Am. Chem. Soc.*, **62**, 2580 (1940).

5% aqueous pyridine adjusted to *p*H 5.0 with sulfuric acid. The lysozyme is subsequently crystallized.

B. Direct crystallization procedure.[3] Crystalline lysozyme having been obtained by the above procedure, the antibiotic may be more conveniently prepared by direct crystallization from egg white. This is done by adding 5% of solid sodium chloride to egg white, adjusting the *p*H to 9.5 with sodium hydroxide, seeding with a small amount of crystalline lysozyme, and allowing the mixture to stand at 4° for 48 hours.

II. Starting Material

Fresh, frozen, or technical egg white may be used. Some difficulty is encountered with low-grade technical egg white due to the presence of excessive amounts of contaminants such as yolk lipids and decomposition products. The egg white is filtered through cheesecloth to remove chalazae and is then homogenized by stirring for 10–15 minutes at a moderate rate with a motor-driven stirrer. Whipping of air into the egg white should be avoided.

III. Procedure

A. Adsorption procedure. To 1 l. of homogenized egg white (3 dozen eggs of average size) are added 150 ml. of a 10% suspension of bentonite (Volclay, American Colloid Company, Chicago) in 1% potassium chloride. The mixture is stirred vigorously (avoid excessive foaming) for 3–5 minutes until a smooth suspension is obtained. The clay is separated by centrifugation (preferably in a Sharples centrifuge) and washed twice with 300-ml. portions of 0.5 *M* phosphate buffer (*p*H 7.5) and three times with 300-ml. portions of 5% aqueous pyridine. The clay is separated by centrifugation after each washing, and the supernatants, containing inactive proteins, are discarded.

Lysozyme is eluted by washing the clay twice with 300-ml. portions of a 5% aqueous solution of pyridine which has been adjusted to *p*H 5 (glass electrode) with sulfuric acid. The elution

[3] G. Alderton and H. L. Fevold, *J. Biol. Chem.*, **164**, 1 (1946).

step should follow the adsorption within 24 hours. The eluates are dialyzed against running tap water (12–15°) until no odor of pyridine remains.[4] The dialysis is completed against running distilled water (12–15°) for 24 hours. The *p*H of the final solution is approximately 6. The volume increases considerably during the dialysis. Amorphous but essentially pure lysozyme is obtained by lyophilization of the solution.[5] The yield is 2.0–2.5 gm. per liter of egg white. Crystalline lysozyme or its salts are obtained from the amorphous material by one of the following procedures.

1. To 10 ml. of a 5% solution of amorphous lysozyme is added 0.5 gm. of sodium bicarbonate (final *p*H 8.0–8.5), and the solution is allowed to stand at room temperature. Lysozyme carbonate crystallizes.

2. To 10 ml. of a 5% solution of amorphous lysozyme is added 0.5 gm. of sodium chloride, and the *p*H is adjusted to 9.5–10.0 with sodium hydroxide. The solution is stored at 4°. Crystalline isoelectric lysozyme separates.

3. A 5% solution of amorphous lysozyme is adjusted to *p*H 4.5 with hydrochloric acid, and 5% of solid sodium chloride is added. The solution is stored at 4°, and crystalline lysozyme chloride is deposited in 4–5 days.

B. Direct crystallization of lysozyme from egg white. Fifty grams of sodium chloride are dissolved in 1 l. of homogenized egg white, and the solution is adjusted to *p*H 9.5 with 1 *N* sodium hydroxide (45–50 ml. are required). A small amount of crystalline isoelectric lysozyme is added with stirring. The mixture is allowed to stand at 4° and is stirred 5–6 times daily until the lysozyme separates (2–4 days). The crystalline material is

[4] The checkers used Viscose tubing, Visking Company, Chicago, for this step.

[5] Since the volume of solution is large (1–1 2 l) the checkers found it convenient to concentrate the solution first by pervaporation (P. A. Kober, *J. Am. Chem. Soc.*, 49, 944 [1917]) The concentration was carried out by suspending the dialyzing bag containing the solution in the moving air before an electric fan for 24 hours. The temperature of the contents of the bag remains below 15° because of the cooling effect of the evaporation of water. The volume of solution was easily reduced from about 1.2 l. to 225 ml. in 24 hours. The solution was then removed from the dialyzing bag, the bag was rinsed out with a little water, and the wash was combined with the concentrated solution and lyophilized.

removed by centrifugation and dissolved in acetic acid (*p*H 4.5). Insoluble material is separated by centrifugation and re-extracted with water. The two extracts are combined, and 5% of solid sodium bicarbonate is added (*p*H 8.0–8.5). Crystalline lysozyme carbonate separates. Or 5% of solid sodium chloride is added and the solution is adjusted to *p*H 9.5–10.0 with sodium hydroxide. Isoelectric lysozyme crystallizes. The yield is about 3.0 gm. per kg. of egg white.

IV. Properties and Purity of Product

Lysozyme has been shown to be a pure protein by sedimentation, diffusion, and electrophoretic criteria.[1] It has a molecular weight of approximately 17,500 and is a basic protein [1,6] with an isoelectric point of 10.5–11.0. Lysozyme contains 20–22 basic groups per mole as compared with only 3–4 acidic groups and therefore forms soluble salts with the common acids. A number of these have been obtained in a crystalline form (chloride, iodide, bromide, carbonate, nitrate).[3] Lysozyme is relatively stable in acid solution [1] but is more labile in alkaline media. Lysozyme contains no phosphorus, 2.3% sulfur, 17.5% nitrogen, 6.74% hydrogen, and 48.7% carbon.

Lysozyme exerts its antibiotic action by hydrolyzing a mucopolysaccharide of the bacterial cell wall.[7] This activity has been utilized as the basis of a viscosimetric assay procedure for lysozyme.[8]

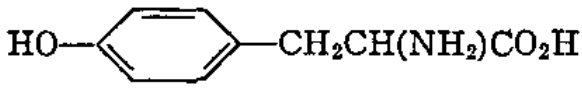
V. Methods of Preparation

Lysozyme has been obtained from egg white by procedures involving solvent fractionation and precipitation as the insoluble flavianate.[9] It has been isolated by adsorption on bentonite.[1] and by direct crystallization from egg white at *p*H 9.0–9.5.[3] Lysozyme occurs in the latex of certain plants, and pure *Ficus*

[6] E. P. Abraham, *Biochem. J.*, 33, 622 (1939).
[7] K. Meyer et al., *J. Biol. Chem.*, 113, 479 (1936).
[8] K. Meyer and E. Hahnel, *J. Biol. Chem.*, 163, 723 (1946).
[9] K. Meyer et al., *J. Biol. Chem.*, 113, 303 (1936).

lysozyme has been isolated and shown to differ chemically from egg white lysozyme.[10] The present procedures are essentially those of Alderton, Ward, and Fevold [1] and Alderton and Fevold.[3]

D-TYROSINE



Mol. wt. 181.19 ($C_9H_{11}NO_3$)

Submitted by ROBERT R. SEALOCK, Iowa State College, Ames.
Checked by F. R VAN ABEELE and H. E CARTER, University of Illinois, Urbana.

I. Principle

The natural isomer, L-tyrosine, is catalytically racemized with excess acetic anhydride in aqueous solution containing sodium acetate. The diacetyl-DL-tyrosine which is produced is hydrolyzed to *N*-acetyl-DL-tyrosine monohydrate. Crystallization of the brucine salts of the latter from ethanol yields the salt of the D-isomer. Upon decomposition *N*-acetyl-D-tyrosine is obtained. Hydrolysis of this compound gives D-tyrosine in an overall yield of 65%.

II. Starting Material

L-Tyrosine obtained from the usual commercial sources and of reasonable purity may be used. The tyrosine residue commonly obtained in the preparation of other amino acids from proteins is satisfactory starting material after one recrystallization from either hydrochloric acid or sodium hydroxide solutions.

III. Procedure

A. N-Acetyl-DL-tyrosine monohydrate. In a 4-l. beaker 181 gm. (1 mole) of L-tyrosine are suspended in 1 l. of water and

[10] K. Meyer, E. Hahnel, and A. Steinberg, *J. Biol Chem.*, 163, 733 (1946)

1 l. of 2 *N* sodium hydroxide (2 moles), exactly standardized against a supply of 6 *N* sulfuric acid, is added. The solution is stirred continuously, and 660 ml. (7 moles) of redistilled acetic anhydride are added in ten 66-ml. portions at 10-minute intervals. The warm reaction mixture is placed in a water bath at 60 to 70° for 6 hours. It is then exactly neutralized with 6 *N* sulfuric acid, and the mixture is concentrated in vacuum. To remove additional acetic acid the distillation is continued with the addition of two 200-ml. portions of water. The thick syrup and sodium sulfate are extracted with one 500-ml. and four 100-ml. portions of acetone. The extracts are combined and evaporated to dryness in vacuum, and the residue is dissolved in 200 ml. of water. The hydrolysis of the O-acetyl group is accomplished by the addition of 800 ml. of 2 *N* sodium hydroxide, the reaction being tested in order to be sure that it is strongly alkaline to phenolphthalein. After 30 minutes at room temperature the calculated amount of 6 *N* sulfuric acid to exactly neutralize the alkali is added.

The solution is concentrated in vacuum, and the residue is extracted with acetone. The extract is concentrated giving crude *N*-acetyl-DL-tyrosine as a thick syrup. This is dissolved in 600 ml. of water, and the solution is allowed to stand in the cold until crystallization occurs (about 24 hours). Occasional stirring promotes more rapid crystallization and yields a material more readily filtered. The yield of crystalline material is 210 gm., and an additional 8.0 gm. are obtained by concentrating the mother liquor to one-fifth volume. The total yield of *N*-acetyl-DL-tyrosine monohydrate (m.p. 94–95° cor.) is 218 gm. (90%).

B. Resolution of N-acetyl-DL-tyrosine. In a 2-l. Erlenmeyer flask are placed 121 gm. (0.5 mole) of N-acetyl-DL-tyrosine monohydrate, 197 gm. (0.5 mole) of anhydrous brucine, and 1.6 l. of absolute ethanol. The mixture is heated to boiling on a hot plate to ensure complete solution. The flask is allowed to stand at room temperature until crystals begin to separate, at which time the solution is well stirred and the container scratched to promote further crystallization. The best yields are obtained if the stoppered container is stored in the cold with occasional stirring for 2 or 3 days. In that time a little more than half of the

total salt is obtained. The crystals are filtered quickly (18.5 cm., Whatman No. 1 filter paper), pressed firmly, and washed with 50 ml. of cold absolute ethanol. The air-dried material should at this stage weigh 165 to 170 gm. The salt is purified by 3 or 4 recrystallizations from 95% ethanol. Equally satisfactory results are obtained from the minimum volume of solvent up to 5 volumes. The recrystallized, air-dried material (m.p. 150–155° with shrinking at 130°) contains from 7 to 12% of water of hydration. Since the completely anhydrous salt is hygroscopic the optical rotation and hydration are obtained by drying approximately 137 mg. in a Fischer drier in vacuum over phosphorus pentoxide at 100°. The dried sample is weighed and dissolved in 95% ethanol, diluted to 25 ml., and the optical activity is determined. A 0.5% solution of the anhydrous salt gives $[\alpha]_D = -42.3°$. An average yield of 268 gm. (75%) of pure anhydrous salt is obtained.

C. **Acetyl-D-tyrosine.** A quantity of the air-dried salt representing 123 gm. (0.2 mole) of the anhydrous compound is dissolved in 20 volumes of water by warming. The solution is quickly cooled to approximately 40°, and 200 ml. of 2 *N* sodium hydroxide are added with thorough stirring. After 12 hours in the cold the brucine tetrahydrate is filtered off and thoroughly washed with 5 portions of cold water. The combined alkaline filtrates are extracted 5 times with chloroform to remove the last traces of brucine. The calculated amount of 6 *N* sulfuric acid is added, and the solution is evaporated to dryness in vacuum. The residue is extracted with acetone, and the acetone solution is concentrated to dryness. The residue is recrystallized from 140 ml. of water, giving 32 gm. (70% yield) of acetyl-D-tyrosine (m.p. 153–154°; $[\alpha]_D = -48.3°$ [0.5% solution in water]). The mother liquors contain acetyl-D-tyrosine and yield appreciable quantities of D-tyrosine if worked up as described in section D.

D. **D-Tyrosine.** Because of the solubility and difficulty of crystallization of acetyl-D-tyrosine, the preferred procedure is to dissolve the acetone-soluble residue of the above paragraph in 440 ml. of 5 *N* hydrochloric acid and hydrolyze without isolation

of the acetyl derivative. Hydrolysis is accomplished by gently refluxing the solution for 2.5 hours. The excess acid is removed by concentrating to dryness in vacuum and redistilling to dryness with 3 portions of water. The residue is dissolved in 400 ml. of water and treated with Norite. The solution is made slightly alkaline with sodium hydroxide and then just acid to litmus with a few drops of acetic acid. After 24 hours in the cold, the precipitated tyrosine is removed by filtration and washed with cold water until free of chloride ion, and then with 2 portions of 95% ethanol and 2 of ether. By this method 32.6 gm. (90% yield) of D-tyrosine are obtained from 0.2 mole of the brucine salt.

IV. Properties and Purity of Product

The D-tyrosine obtained in this preparation gives the correct analytical data and has a rotation of $[\alpha]_D^{25} = +10.3$ (4% solution in 1 *N* hydrochloric acid). Sealock [1] has summarized the properties of the N-acetyl and O,N-diacetyl derivatives of L-, D-, and DL-tyrosine.

V. Methods of Preparation

D-Tyrosine has been prepared by the resolution of N-benzoyl-DL-tyrosine,[2] formyl-DL-tyrosine,[3] and N-acetyl-DL-tyrosine [1] through the brucine salts. It has also been reported [3] that the action of pancreatic extract on DL-tyrosine ethyl ester results in the preferential hydrolysis of the L-ester.

[1] R. R. Sealock, *J. Biol. Chem.*, 166, 1 (1946).
[2] E. Fischer, *Ber.*, 32, 3638 (1899).
[3] E. Abderhalden and H. Sickel, *Z. physiol. Chem.*, 131, 277 (1923).

SUBJECT INDEX

The titles of individual preparations are given in SMALL CAPITAL LETTERS. Volume numbers are indicated by ordinary **bold-face** type. Preparations or procedures for which specific laboratory directions are given are denoted by ***bold-faced italics;*** substances which are only mentioned with no experimental detail are indicated by ordinary type.